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Bacterial Bioassay for Level I Toxicity Assessment

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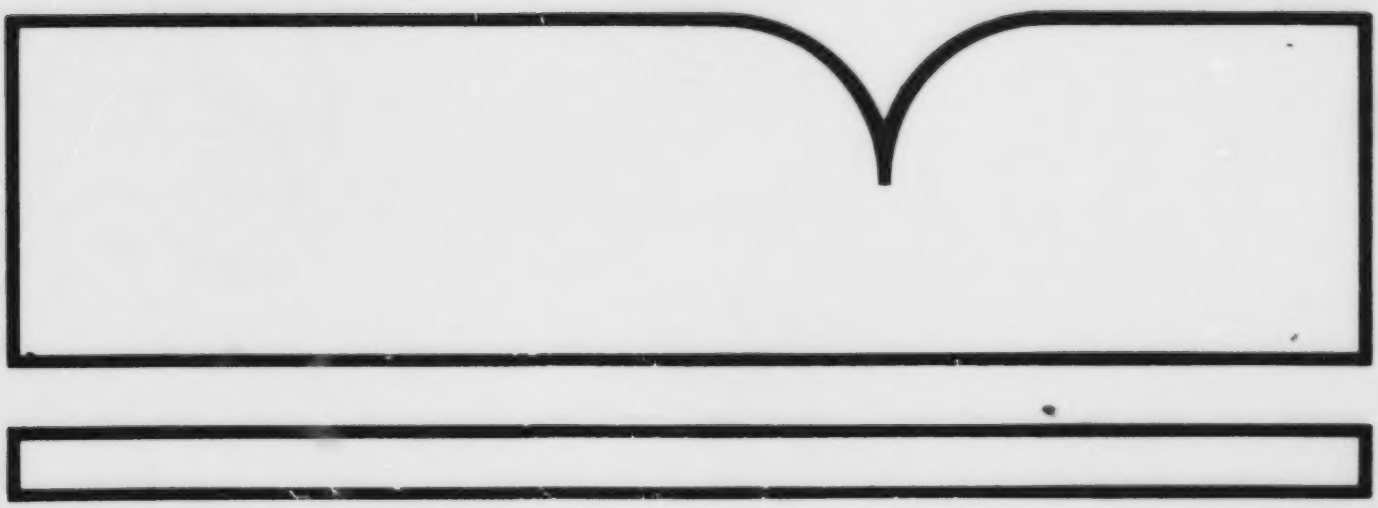

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BACTERIAL BIOASSAY FOR LEVEL I TOXICITY ASSESSMENT

by

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The toxicity of five metals and three organic toxicants to the nitrifying bacteria (Nitrobacter and Nitrosomonas) were determined and compared to other common bioassay organisms. In general, bacterial exhibited somewhat lower sensitivity for general metabolic toxicants, but dramatically lower sensitivity for specific target-site toxicants.

The application of the bacterial bioassay was shown for two cases of Level I testing: a field study of a toxic industrial waste and its pre-treatment and an assessment study of the potential leachate problems for a flue-gas scrubber solid waste.

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SECTION 1

INTRODUCTION

BACKGROUND

Bioassay tests can be used to detect biologically harmful chemicals whose effects can be manifested as cellular, genetic, behavioral, or metabolic damage. Many different bioassay tests are presently used by health officials to detect toxic chemicals (1)(2). For example, the Environmental Protection Agency (EPA) has developed a three-phased approach to performing assessment of the toxicity of aqueous solutions (including solid waste leachates); this program is divided into Level 1, 2, and 3 tests (3)(4)(5). Level 1 involves general bioassay and analysis procedures that will identify the presence of toxicity in a given waste stream. Level 2 tests are used to identify and quantify specific compounds associated with the toxicity found in the Level 1 test. Level 3 tests will provide more detail concerning chronic health and ecological effects of the stream components.

The focus in the Level 1 tests is a complementary series of bioassay tests. These tests and their brief description are listed in Table 1 (6). These tests provide no specific identification of the toxicant, but serve as signals for a wide range of potentially toxic responses.

The primary difficulty with the application of these tests is the complexity that results in high costs. The tests require highly trained personnel, modern laboratories, and long time periods. A listing of estimated unit costs for these tests are included in Table 1 (7). Total costs could exceed $5000 per sample for all the tests. These costs appear reasonable for a small number of samples. However, in situations where wastewater compositions vary temporally or with process changes the cost of this assessment program could be prohibitive.

An attractive alternative to the bioassay organisms listed in Table 1 for acute toxicity is lower level organisms, especially bacteria. Such a bacterial bioassay would be supplemental to the proposed Level 1 bioassay tests and other health and toxicity tests and, hopefully, correlative. The advantage of using bacteria as compared to the other Level 1 bioassay organisms for acute toxicity would be greater simplicity, shorter testing times, and lower cost. Such a test could be accomplished within a few hours by chemical technicians and would involve minimal laboratory facilities.

If a bacterial bioassay could identify the presence of a wide range of toxic compounds, it could be widely used for routine and continuous monitor-

TABLE 1. LEVEL 1 - BIOASSAY TESTS (AFTER 6).

Bioassay System	Test Organism	Purpose of Test	Unit Cost (7)
Microbial mutagenicity	Nine different strains of bacteria and one of yeast.	To determine if a chemical mutagen (possibly a carcinogen) is present.	$ 350
Cytotoxicity	Rabbit alveolar cells and Chinese hamster ovary cells.	To measure metabolic impairment and death in mammalian cells.	250
Freshwater and marine static bioassay	Fathead minnow, daphnids, sheepshead minnows, and grass shrimp.	To detect potential toxicity to organisms present in aquatic environments.	1,250
Freshwater and marine algal assay	Freshwater and marine algae.	To detect potential growth inhibition and stimulation effects on primary producers.	1,373
Range finding acute toxicity	Young adult rats.	Whole animal test to detect potential toxic effects to mammals.	330
Terrestrial ecology	Soil microorganisms.	To determine potential inhibition and stimulation effects on soil microorganisms. These data are useful if the effluent is used for crop irrigation.	1,280
		TOTAL COST	$5,348

ing, and assessment of a wide number of waste streams. It potentially could be used to survey wastewaters discharged to fresh and marine environments, to public and private treatment facilities, and to terrestrial environments. The test could potentially identify industrial wastes requiring pretreatment and/or compliance with pretreatment standards (8). In addition, this test could identify hazardous solid wastes by detection of toxicity in leachates (9). Due to its low costs, the test could be used to identify which chemical or physical fraction of a toxic wastewater contained the toxicant (1). The need definitely exists for a simple, rapid, low cost bioassay test and a bacterial bioassay potentially could fulfill this role. Previous researchers (10)(11)(12)(13) have shown the applicability of bacterial bioassays using nitrifiers, marine luminescent bacteria, and mixed heterotrophs for a wide range of toxicants.

Initial Studies

Williamson (10) has reported initial development of a bacterial bioassay test using Nitrobacter. These organisms are strict aerobes which obtain their energy from the oxidation of nitrite to nitrate. Carbonate is their sole carbon source. These organisms were chosen over other heterotrophic bacterial species such as used by Bauer, Seidler, and Knittel (13) since the cells can be grown in a simple aqueous medium of nitrite, oxygen, carbonates, phosphorous, and basal salts and, therefore, the growth medium does not contribute organics to the bioassay test. In addition, these autotrophic organisms do not metabolize organic toxicants. The growth medium has low levels of inorganic salts as opposed to the high levels in the bacterial luminescence assay developed by Bulich (11) and Bulich, et al. (12) and thus complexation of metal toxicants is avoided. Methods were developed to freeze-dry and reconstitute Nitrobacter so that standard organisms could be used by a wide number of laboratories. The results of this work were promising enough to warrant further research to determine the applicability of the autotrophic nitrifying bacteria as test organisms for a Level 1 bacterial bioassay.

SECTION 2

CONCLUSIONS

Nitrifying bacteria can be used as Level 1 bioassay organisms to detect acute toxicity of aqueous solutions. The bacterial bioassay is simple, rapid, and low cost and could be standardized using freeze-dried organisms. It may be useful in cases where comparative toxic levels of a large number of samples are required, or for field application with limited equipment and personnel.

The bacteria bioassay with either Nitrobacter or Nitrosomonas exhibits somewhat lower sensitivities for general metabolic toxicants (such as heavy metals) compared to other common bioassay organisms, but exhibits dramatically lower sensitivity for specific toxicants (such as pesticides).

The bacterial bioassay can be used to determine relative toxicities of aqueous solutions to verify theoretical models of chemical composition (e.g., toxicity of cadmium speciation), to optimize treatment of toxic wastewaters, or to assess toxicity of solid waste leachates under various leaching conditions. A continuous-flow bacterial bioassay can be used to continuously monitor for toxic discharges from point sources.

SECTION 3

RECOMMENDATIONS

The bacterial bioassay should be considered for adoption as a Level 1 bioassay test by the EPA. The EPA could develop facilities to provide freeze-dried nitrifying organisms to the public for such testing.

The continuous-flow bacterial bioassay should be further developed to detect discharges of toxic wastes from selected point sources. Such testing could substantially reduce toxic loads to municipal treatment facilities and the nation's waterways.

SECTION 4

RESEARCH PLAN

From the initial studies with Nitrobacter, two important considerations for further research were identified. First, it was questioned whether the similar autotrophic organism Nitrosomonas also could be used as a test organism in addition to Nitrobacter. Several studies had suggested that some chemicals that had low toxicity to Nitrobacter had a higher sensitivity to Nitrosomonas. Second, it was unknown what the relative sensitivity of these two bacterial genera were to other bioassay organisms. A comparable sensitivity to other organisms would be required for adoption of a bacterial bioassay.

Based on these two questions and other consideration for application, the objectives of this study were developed as:

1. Develop methods to successfully freeze-dry Nitrosomonas.

2. Determine the toxic concentrations of a wide range of known toxicants to Nitrobacter and Nitrosomonas.

3. Compare the concentrations from Objective 2 to known toxic levels for other health and ecological bioassays presently used.

4. Develop and describe the use of nitrifying bacteria to estimate toxicity and the type of toxicant present in complex wastewaters or leachates.

5. Develop a continuous, on-line monitoring system using nitrifying bacteria to determine the presence of toxic materials in wastewater streams and/or compliance with pretreatment standards.

APPROACH

To fulfill the objectives described, the project was divided into five research tasks:

1. A literature review of the toxic levels to common bioassay organisms of several organic and inorganic toxicants.

2. Determination of the toxic levels of these same organic and inorganic toxicants to Nitrobacter and Nitrosomonas.

3. Development of freeze-drying techniques for Nitrosomonas.

4. Determination of toxic response to Nitrobacter of complex wastewaters of two types of toxicants: an industrial waste from a fiberboard manufacturer and a simulated leachate from a solid waste.

5. Development of a continuous-flow technique using specific ion electrodes to continuously monitor for a toxic response.

A description of the experimental procedures and results for each of these phases are contained in the following sections.

SECTION 5

TOXICITY TO NITRIFYING BACTERIA

Numerous studies have been conducted on the mechanisms of toxicity to bacteria including the nitrifiers. In this section this information is reviewed to allow assessment of what types of toxic chemicals could probably be detected with the proposed nitrifying bioassay.

TOXIC MECHANISMS TO BACTERIAL CELLS

Several different mechanisms exist by which a chemical agent can disrupt the cell function such as catalytic participation, structure stabilization, or disruption or modification of biochemically-poised structures such as proteins and membranes. A summary of different mechanisms is presented in Table 2. Of these toxic mechanisms, all except the pesticides and cyclodienes are expected to exhibit specific toxicity to bacteria including the nitrifiers since bacteria lack acetylcholinesterase (62).

KINETIC THEORY OF TOXICITY

Hartmann and Laubenberger (15) gave a detailed summary of a biochemical theory for bacterial toxicity. The theory is based on enzyme inhibition by the toxic material; the kinetics of substrate utilization are assumed to follow Michaelis-Menten or Monod relationships as:

$$r = -k \frac{S}{S+K_s} X, \qquad (1)$$

where r = rate of substrate utilization,

k = maximum substrate utilization rate,

S = limiting substrate concentration,

X = bacterial concentration, and

K_s = half-velocity coefficient.

TABLE 2. VARIOUS MECHANISMS OF TOXICITY

Inhibitor	Toxicity Mechanisms
Heavy Metals (general)	(1) interference with cell wall synthesis (2) decreased oxidative enzyme activity (3) deactivation of DNA, RNA, and proteins
Specific Heavy Metals	
Hg	(1) reacts with sulfhydryl (SH-) groups of the cells decreases enzyme activity
Ag	(1) formation of metal complexes with polynucleotides, DNA, RNA
Cu	(1) binds with DNA (2) coagulation of bacterial cell colloid
Zn	(1) complexation with essential nutrients
Phenols	(1) disrupt cell membranes (2) inhibition of oxidase enzymes (3) protein precipitation inside the cells
Alcohol	(1) inhibition of respiration (2) inhibition of phosphorylation (3) damage to the cell membrane
NH_4^+-based Compounds	(1) inhibition of bacterial oxidases (2) inhibition of dehydrogenase system (protein denaturation and enzyme suppression)
Chlorine	(1) attacks sulfhydryl (-SH) groups of enzymes involved in metabolic pathways
Acid (H^+) or Base (OH^-)	(1) displaces ion species such as Na^+, Ca^{++} from absorption sites of the cells (2) cell wall damage
Organophosphate Pesticides	(1) inhibition of acetylcholinesterase (2) respiratory toxicity, high dermal toxicity
Carbamate Pesticides	(1) inhibition of acetylcholinesterase
Organochloride Pesticides	(1) actions on nerve fibers; activities in the nerves still unknown (2) inhibits Na^+, K^+, Mg^{++}, ATP
Chlorinated Cyclodienes	(1) neurotoxicity mechanism unknown

Enzyme inhibition can be identified as competitive or noncompetitive. Competitive inhibition requires the interaction of an inhibiting or toxic agent, I, with the enzyme and the enzyme-substrate complex, to form an enzyme-inhibitor complex. The resulting Michaelis-Menten relationship is:

$$r = -k \frac{S}{S+K_c} X \quad , \tag{2}$$

where K_c = "half velocity" coefficient with competitive inhibition.

Due to inhibition, K_c will always be larger than K_s. With noncompetitive inhibition, the inhibitor can react with the enzyme to form an inhibitor-enzyme complex and with the enzyme-substrate complex to form an enzyme-substrate-inhibitor complex. The appropriate Michaelis-Menten relationship is:

$$r = -k_n \frac{S}{S+K_s} X \quad , \tag{3}$$

where k_n = maximum rate of substrate utilization with noncompetitive inhibition. Due to inhibition, k_n will always be less than k.

Typically a toxic agent will result in both competitive and noncompetitive inhibition. In the presence of a toxicant, the nitrifying bioassay should detect either competitive and/or noncompetitive inhibition since the K_s values for both NO_2^--N and NH_4^+-N are less than 1 mg/ℓ (16).

CHEMICALS CAUSING NITRIFICATION INHIBITION

Nitrifying bacteria can also be significantly affected by toxic substances in the aqueous environment. Numerous researchers have investigated the effects of chemicals on biological nitrification (17-24) which is summarized below.

A variety of materials, including heavy metals, chlorates, cyanides, alkaloids, mercaptans, urethanes, quanidines, methylamines, nitrourea, thiourea, phenolic compounds, cresols, halogenated solvents, chelating agents, and certain fatty acids, can be toxic to nitrifying organisms (22). Inhibitory concentrations of various metals and organics are listed in Table 3. Specific inhibitor may result in death of the nitrifying bacteria, in temporary reduction of metabolism with resumption of normal nitrification rates after removal of the inhibitor, or in only a decrease in the growth rate. The effects of inhibitors vary between Nitrosomonas and Nitrobacter with Nitrosomonas more sensitive to most toxicants.

TABLE 3. INHIBITORY CONCENTRATION FOR BIOLOGICAL NITRIFICATION (22)

Inhibitor	Toxic Levels (mg/ℓ)
Cobalt	59.0
Copper	20.0
Chromium	0.25
Mercury	2.0
Nickel	11.7
Silver	0.25
Zinc	3.0
Thiourea	0.076
Thioacetamide	0.53
Aniline	7.7
Guanidine Carbonate	16.5
Phenol	5.6
Sodium Cyanide	0.65
Ally Alcohol	19.5
Methyl Isothiocyanate	0.80
Mercaptobenzothiazole	3.0

Lees and Simpson (24) studied the effects of chlorates, chlorite and cyanate on nitrite oxidation by Nitrobacter. They proposed a specific biochemical mechanism for the effects of inhibitors on nitrite oxidation. Cyanate inhibited nitrite oxidation, but the effect was reversible. Chlorate caused a gradual decrease in the rate of nitrite oxidation and was irreversible. Cytochrome 551, an important nitrite oxidizing enzyme, is destroyed by chlorite, explaining its inhibitory action.

The effects of copper, nickel, zinc, a fungicide, and a wastewater on both carbonaceous oxidation and nitrification in activated sludge units are studied by Green, et al. (18). Significant decreases in nitrification were noted with the metals, the fungicide, and the wastewater. Jackson and Brown (20) presented a comprehensive review of metals and organics that are toxic to aerobic treatment processes. Cadmium was nontoxic at concentrations below 5 mg/ℓ. They compared toxicity levels of heterotrophic bacteria to autotrophic nitrifiers and demonstrated the sensitivity of nitrifying bacteria to toxic materials.

Tomlinson, et al. (23) developed a simple short-term test for inhibition of nitrification in activated sludge. This method was applied in screening numerous organic compounds and several metals for inhibition of ammonia oxidation. Metals were must less toxic to nitrification in activated sludge cultures than in pure cultures. Complexing of metals with organic matter in sludges is a likely explanation. Downing, et al. (17) used a similar procedure to screen numerous organics for toxicity to nitrification. Hockenbury and Grady (19) also studied the inhibition of nitrification by selected organic compounds. Nitrosomonas was much more sensitive to the compounds tested than Nitrobacter. High ammonia concentrations were found to be inhibitory to nitrite oxidation by Nitrobacter. The effects of phenols and heterocyclic bases on nitrification were investigated by Stafford (21). Phenol inhibited ammonia oxidation at 4 to 10 mg/ℓ, but levels as high as 100 mg/ℓ failed to affect nitrite oxidation. On the other hand, pyridine completely inhibited Nitrobacter at 100 mg/ℓ, whereas Nitrosomonas was only partially inhibited. Holland and Green (25) also reported on the inhibition of ammonia oxidation by metals and organics.

In general, these reported results for the toxic response of the nitrifiers suggest that these organisms could detect a wide range of both organic and inorganic toxicants. Few compounds show a specific toxicity to nitrifiers that would not induce a toxic response in other organisms, so the potential for "false-positive" results does not appear significant.

SECTION 6

NITRIFYING BIOASSAY

The nitrifying bioassay, as proposed, uses cultures of either Nitrobacter or Nitrosomonas - Nitrobacter as the test organisms. In this section, the methods for the bioassay test will be described and results from bioassays using eight chemicals given. The eight chemicals were chosen to provide a wide range of inorganic and organic toxicants and included lead, cadmium, copper, zinc, silver, heptachlor, endosulfan, and parathion. The relative sensitivitiy of the nitrifying organisms to these chemicals is compared to other test animals used in standard bioassay tests in Section 7.

BIOCHEMISTRY OF NITRIFYING BACTERIA

Autotrophic nitrifying bacteria derive their energy from the aerobic conversion of nitrogen in the form of ammonium to nitrite to nitrate as:

Nitrosomonas

$$NH_4^+ + 3/2\ O_2 \rightarrow NO_2^- + 2H^+ + H_2O \qquad (4)$$

Nitrobacter

$$NO_2^- + 1/2\ O_2 \rightarrow NO_3^- \ . \qquad (5)$$

Carbon dioxide (or carbonate in the aqueous system) is the sole carbon source for the organisms. The equations for energy yielding reactions can be combined with equations for organism synthesis to form overall synthesis-oxidation relationships. Equations for synthesis-oxidation, using representative measurements of yields to the generalized cell composition of $C_5H_7NO_2$ and oxygen consumptions are (26):

Nitrosomonas

$$55\ NH_4^+ + 76\ O_2 + 109\ HCO_3^- \rightarrow C_5H_7NO_2 + 54\ NO_2^- + 57\ H_2O + 104\ H_2CO_3 \quad (6)$$

Nitrobacter

$$400\ NO_2^- + NH_4^+ + 4\ H_2CO_3 + HCO_3^- + 195\ O_2 \rightarrow C_5H_7NO_2 + 3\ H_2O + 400\ NO_3^- . \quad (7)$$

Experimental cell yield values ranged from 0.04 to 0.13 g volatile suspended solids (VSS) per g ammonia-nitrogen oxidized for Nitrosomonas, and from 0.02 to 0.07 g VSS per g of nitrite-nitrogen oxidized for Nitrobacter (26). Thermodynamic calculations produce values of 0.29 and 0.084 for Nitrosomonas and Nitrobacter, respectively (26).

The substrate utilization rate of the nitrifying bacteria have been found to conform to the Monod relationship (16,26,27) as shown in Eq. (1). Williamson and McCarty (16) measured half velocity coefficients for Nitrobacter and Nitrosomonas - Nitrobacter of 0.07 mg NO_2^- - N/ℓ and 50 mg NH_4^+ - N/ℓ, respectively. The maximum substrate utilization rate ranged from 1 to 4 mg N/mg total suspended solids (TSS) - day.

Because the substrate concentration in a bioassay test will always be greater than the K_s value, the substrate utilization rate (r) will be relatively constant and equal to -kX. The growth rates as shown by the cell yield values are very low. Therefore, either organism is quite suitable for batch bioassay tests because of the relatively linear uptake of substrate over test periods of several hours since X will not change significantly from metabolism of a few mg/ℓ of nitrogen.

Numerous investigators have studied and reported on the parameters that affect the inorganic metabolism of nitrifying bacteria (24,28-32). Lees and Simpson (24) did the classic study on the biochemistry of nitrite oxidation; they found that the cytochromes were intimately involved in the oxidation of nitrite to nitrate. Furthermore, the study suggested that inhibitors act by destroying the required cytochrome. Painter (29) presented a comprehensive literature review on inorganic nitrogen metabolism in microorganisms. He reported that nitrifying bacteria were extremely susceptible to toxicants. The effects of environmental conditions, such as pH, temperature, and dissolved oxygen, were also reported.

Nitrifying organisms prefer alkaline pH and were found to grow best at pH values ranging from 6.3 to 9.4 (29). Wong-Chong and Loehr (32) found an optimum pH for Nitrobacter of 7.3.

Temperature also affects the metabolism of the organisms, although reported optimum ranges vary widely (29). In general, the optimum growth temperature is within the range of 25 to 40°C.

Studies on the effect of dissolved oxygen (DO) concentration on the growth of nitrifiers have shown varying results (26,29). Loehr (33) showed that DO for nitrification is not limiting above 1.0 mg/ℓ. In the bioassay, the DO level was maintained above 2 mg/ℓ to assure that oxygen was not limiting.

PROCEDURE

Culturing of Nitrobacter

Enriched cultures of Nitrobacter and Nitrosomonas - Nitrobacter were grown in a down-flow column similar to the procedure used by Williamson and McCarty (16) (Figure 1). The 15-cm by 72-cm columns were packed with polyethylene beads to prevent washout of cells. Feed for the organisms consisted of 30 mg/ℓ nitrite-nitrogen (as $NaNO_2$) or 10 mg/ℓ ammonia-nitrogen (as NH_4SO_4), 0.2 mg/ℓ phosphorous (as K_2HPO_4), and 20-30 mg/ℓ oxygen in Corvallis tap water, which supplied the micronutrients and bicarbonate for growth. The pH of the feed solution was maintained around 7.0 The column temperature ranged from 20 to 30°C.

Bacteria were removed from the taps on the column in a slurry of organisms and beads which separated readily in a separatory funnel. The organisms flocculated and were concentrated by settling in the bottom of the funnel; the beads were returned to the column.

Freeze-Drying

Freeze-drying provides a means to preserve organisms indefinitely to allow storage and shipment without special handling such as refrigeration. Successful freeze-drying of a microorganism depends first on the proper selection of a cryogenic protective agent. Although much work has been done on freeze-drying bacteria, no work has been reported for Nitrobacter or Nitrosomonas. The most successful method found was to freeze the organisms rapidly in a dry ice-acetone bath in a solution of 10 percent sucrose and 1 percent gelatin. Slower methods apparently lysed the bacteria with large ice crystals, and consequently no survival was obtained.

The bacteria were removed from the column and washed in glass-distilled water and centrifuged to remove excess water. They then were suspended in an approximately equal volume of the cryogenic protective agent of a solution of 10 percent sucrose and 1 percent gelatin. Two mℓ of this suspension then was pipetted into each of several 20 mℓ glass ampules. The ampules were then frozen in an acetone-dry ice bath, attached to a freeze-dryer which removed the water from the frozen suspension, sealed, and stored.

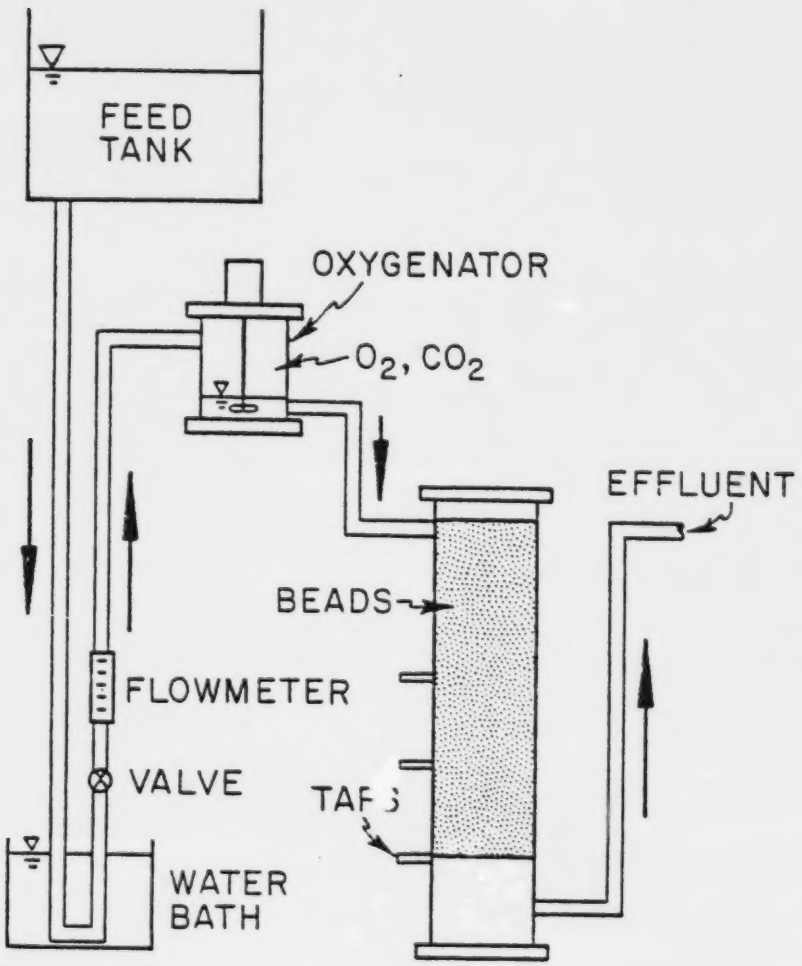

Figure 1. Apparatus for Culturing Nitrifying Bacteria.

Rehydration and resuspension was done with a solution of approximately 5 mg NO_2-N/ℓ for Nitrobacter and 5 mg NH_4^+-N/ℓ for Nitrosomonas. Each ampule was broken open and 2 mℓ of the solution was added dropwise. After the cells were resuspended, they were washed twice to remove as much of the protective agent as possible. The cells then were ready to be used for toxicity testing.

Bioassay

Nitrobacter -- The bioassay procedure for Nitrobacter cultures was as follows:

1) Fifty mℓ of the desired concentration of $NaNO_2$ (100 mg/ℓ NO_2^--N for long-term test and 15 mg/ℓ for short-term test) was prepared with the selected concentrations of toxicants and was placed in a 250 mℓ Erlenmeyer flask. Another 50 mℓ of the same concentration of nitrite solution was placed into a similar flask as the control. The metal toxicants were added as lead nitrate, silver nitrate, cadmium chloride, copper nitrate, and zinc chloride; the toxic organics were all analytical grade (Polyscience Corp.). pH was adjusted to 7.0.

2) An equal volume of Nitrobacter suspension was placed in each flask and the flasks were shaken at a constant temperature for several hours. The cells suspension was added to give an initial absorbance reading in a 1-cm cuvette of from 1 to 2.

3) One mℓ of each solution was taken out periodically and measured for nitrite colorimetrically (34).

4) Dry-weight of the biomass was determined at the end of the experiment by filtration of the solution and by drying at 105°C for one hour.

Nitrosomonas - Nitrobacter -- The bioassay procedure used for Nitrosomonas - Nitrobacter was similar to that for Nitrobacter except that the pH of all solutions were maintained at 7.5. In Step 1 the solutions were prepared with $(NH_4)_2SO_4$ stock solutions and in Step 4, NH_4^+ uptake was measured colorimetrically by the brucine method (34).

DATA ANALYSIS

NO_2^--N or NH_4^+-N concentrations versus time for each flask were plotted and the slope determined by a best squares fit; an example plot is shown in Figure 2a for 2,4,5-trichlorophenol (TCP). A comparison of the calculated slope of each line with the slope obtained for the control yielded the relative metabolism rate (relative rate of either NO_2^--N or NH_4^+-N oxidation) of the test solution. These rates are plotted versus concentration of the wastewater or toxicant; a reduced rate of metabolism confirms a toxic response from the wastewater sample. This is shown for TCP in Figure 2b.

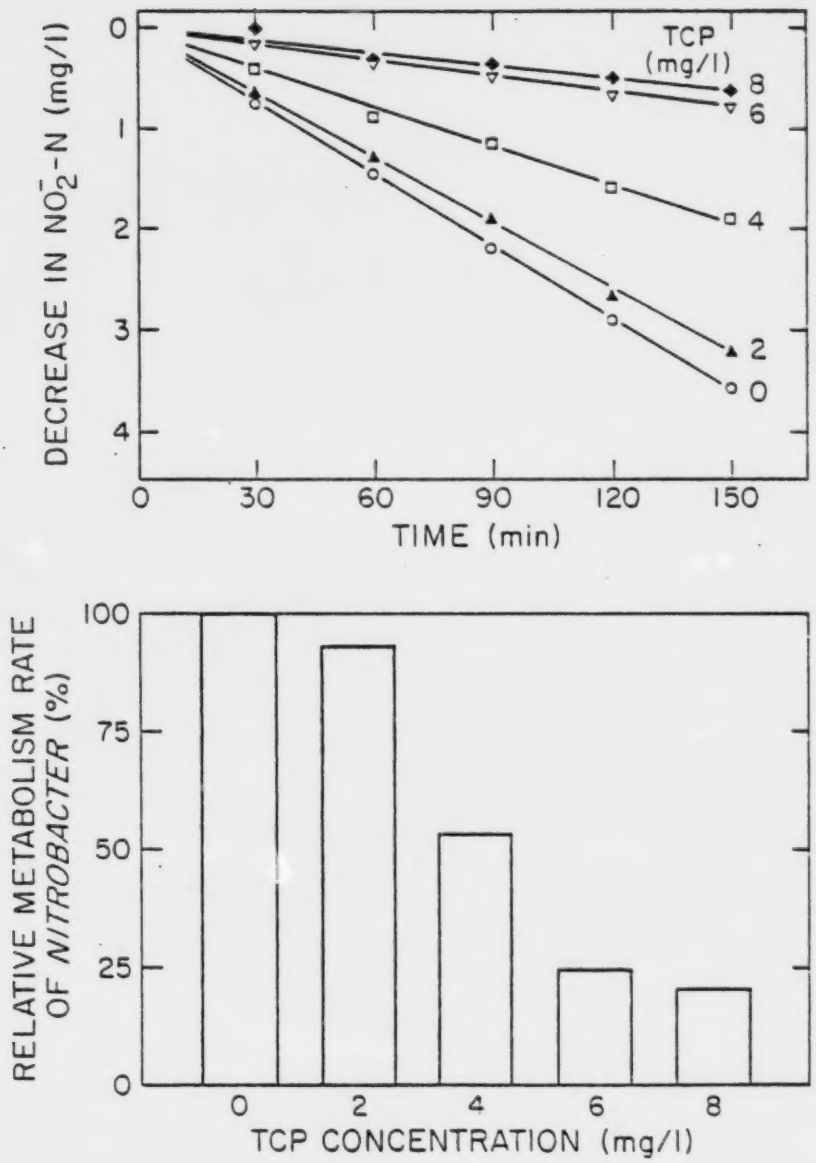

Figure 2. (a) TCP Toxicity Measured by Reduced Substrate Utilization and (b) the Relative Metabolism Rate of Nitrobacter.

RESULTS

Nitrobacter Bioassay

The dose-response curves for Nitrobacter from the eight toxicants are shown in Figures 3 and 4. No significant inhibition was obtained for parathion except at values above 20 mg/ℓ which is the solubility limit. The extrapolated 50 percent and 90 percent relative-metabolism levels are shown in Figure 5; it was judged that these values would represent the range of maximum sensitivities that could be expected in routine application of the bacterial bioassay. These ranges varied for the metals from about 10^{1} to 10^{2} µg/ℓ for silver to 1×10^{5} to 1×10^{6} µg/ℓ for zinc, and from 10^{4} to 10^{5} µg/ℓ for the organics.

All of the results in Figures 3 and 4 were for a 4-hour exposure. An experiment was conducted with cadmium to determine how much the sensitivity could be increased by longer exposure. The results of increasing the exposure to 24 hours are shown in Figure 3. The 50 percent relative metabolism rate was reduced from 4×10^{-4} moles/ℓ for 4 hours to 6×10^{-5} moles/ℓ for 24 hours. Of course, the increased exposure time would result in greater cost of test analysis.

Nitrosomonas Bioassay

The dose-response curve for Nitrosomonas for seven of the eight toxicants are shown in Figures 6 and 7. Only a slight inhibition was obtained for parathion. The 50 percent and 90 percent relative metabolism rates were extrapolated to estimate the range of maximum sensitivity and are shown in the bar graphs in Figure 8. The ranges were similar to the values obtained for Nitrobacter for lead, cadmium, copper, and silver; however, Nitrosomonas exhibited increased sensitivities for zinc, endosulfan, heptachlor, and parathion.

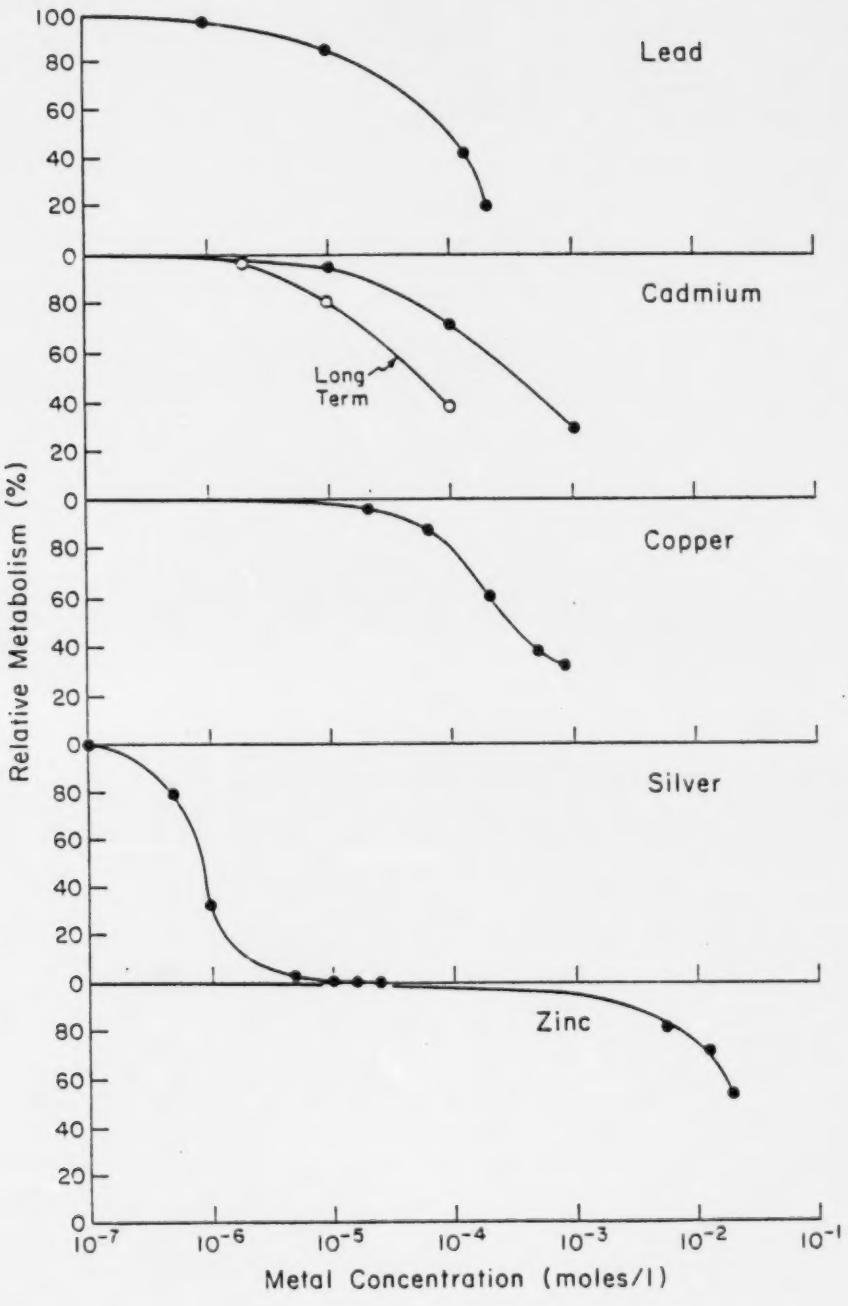

Figure 3. Relative Metabolism of Nitrobacter versus Dosage of Lead, Cadmium, Copper, Silver and Zinc.

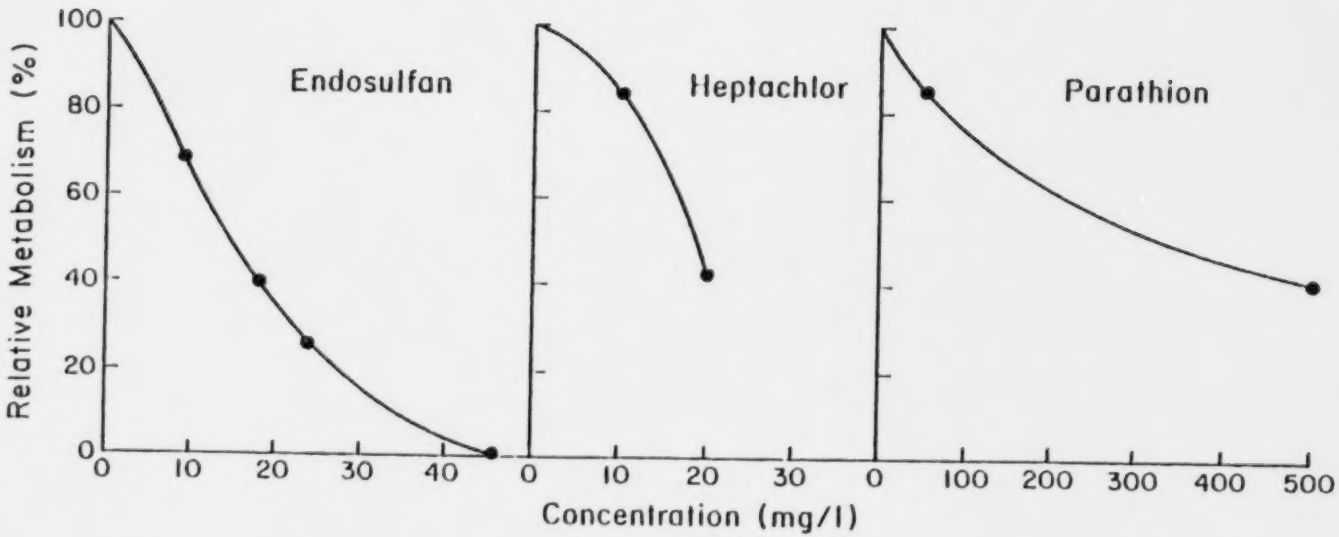

Figure 4. Relative Metabolism of Nitrobacter versus Dosage of Organic Toxicants.

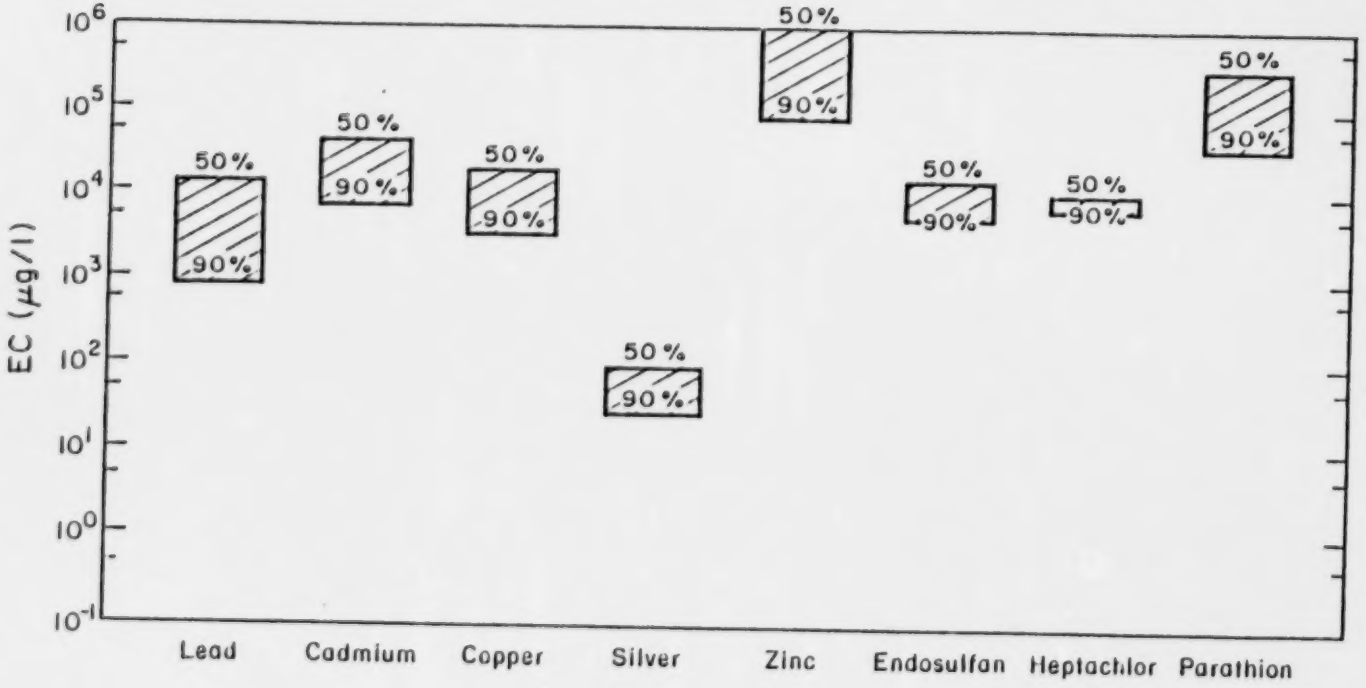

Figure 5. Summary of Relative Metabolism of Toxicants to Nitrobacter.

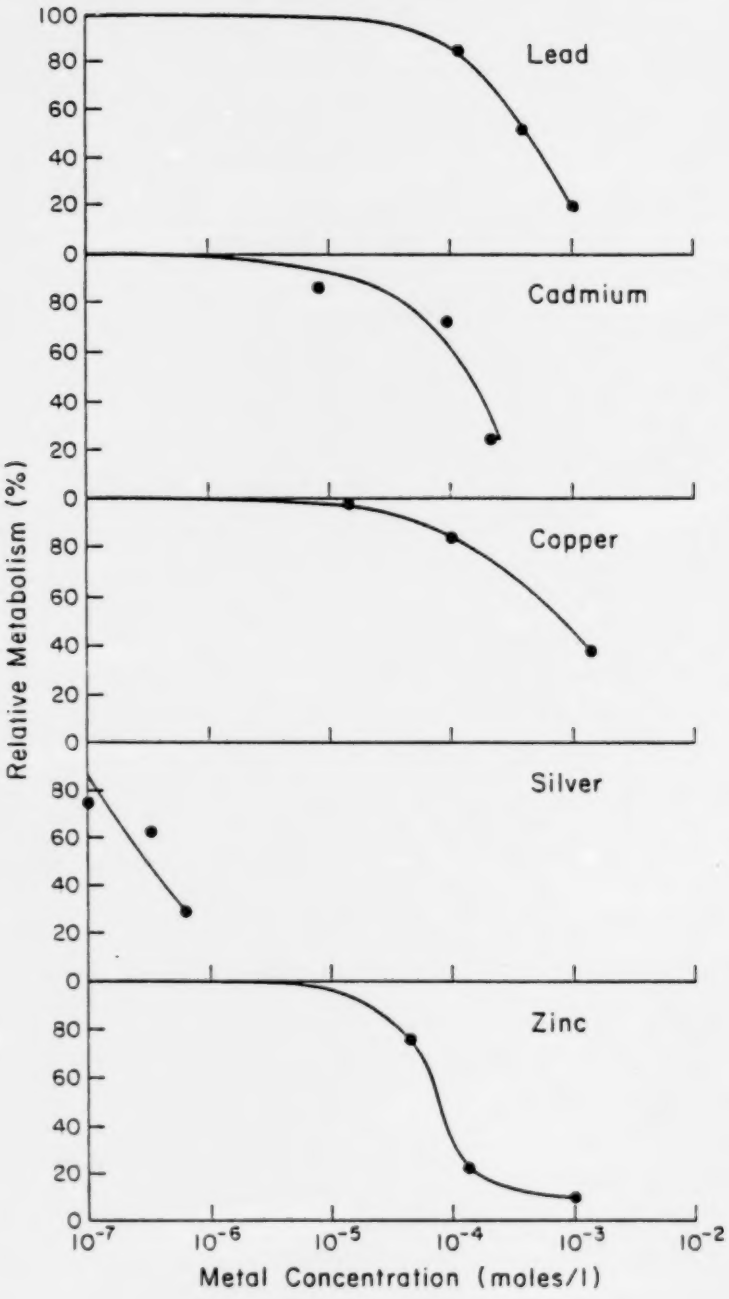

Figure 6. Relative Metabolism Rate of Nitrosomonas versus Dosage of Lead, Cadmium, Copper, Silver, and Zinc.

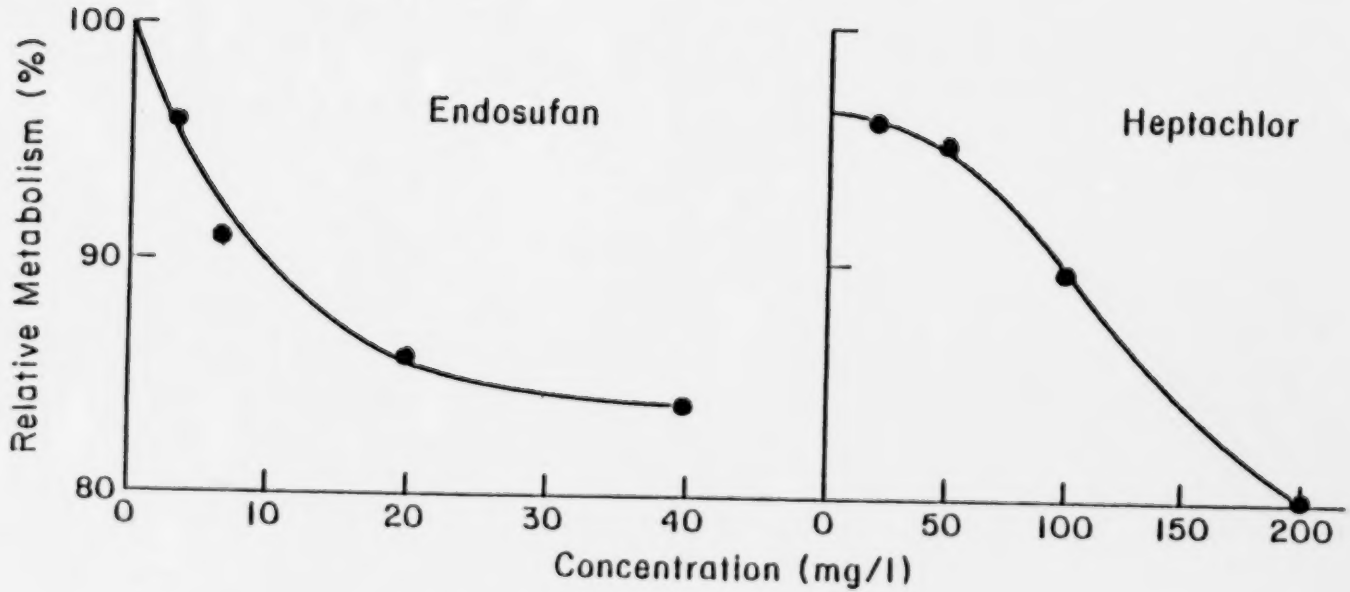

Figure 7. Relative Metabolism of Nitrosomonas versus Dosage of Organic Toxicants

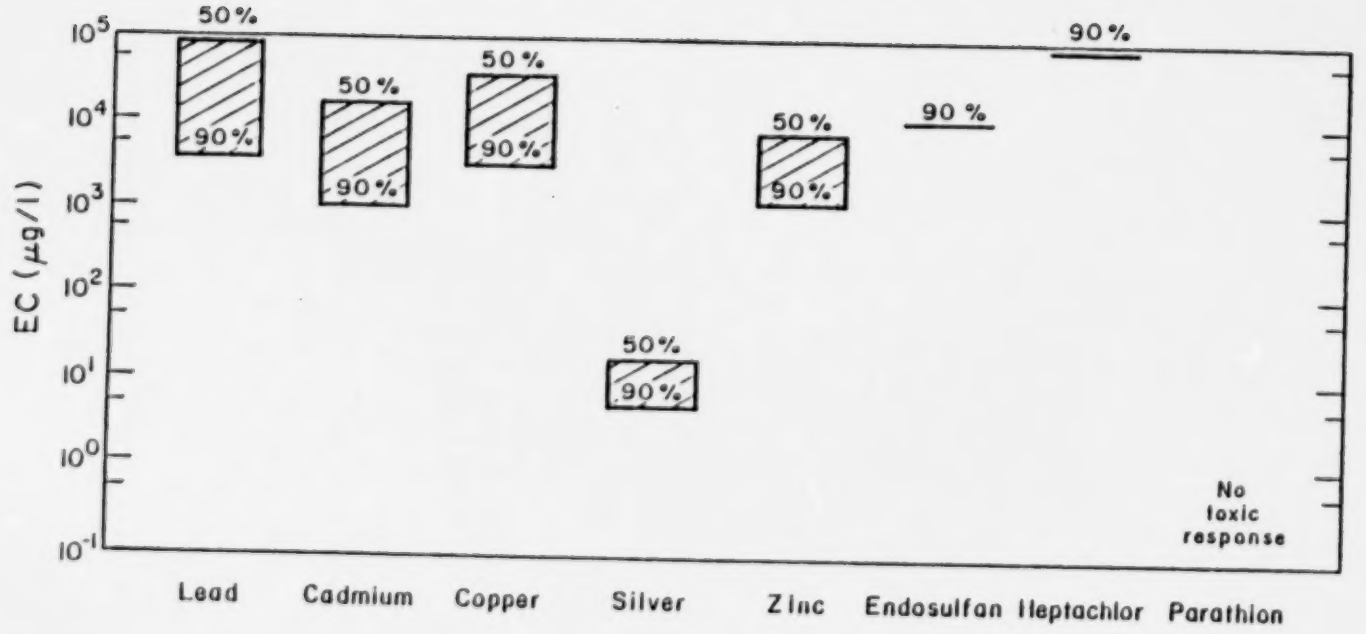

Figure 8. Summary of Sensitivities of Toxicants to Nitrosomonas.

SECTION 7

ACUTE TOXICITY OF SELECTED CHEMICALS TO COMMON BIOASSAY ORGANISMS

In this section the acute toxicity of eight chemicals are summarized and these values compared to the toxic concentrations measured for the nitrifying bacteria (Section 6). The eight chemicals were to provide a wide range of inorganic and organic toxicants and included lead, cadmium, copper, zinc, silver, heptachlor, endosulfan, and parathion. The majority of the literature has been compiled in a series of reports by the Office of Water Regulations and Standards, EPA, under the titles "Ambient Water Quality Criteria for ..." Appropriate citations are made to these references which are available for all eight chemicals except parathion.

LEAD

The toxicity of lead in water, like that of other heavy metals, is affected by pH, hardness, organic materials, and the presence of other metals. Maximum and minimum range values of the LC_{50}/EC_{50} levels for Daphnia, two vertebrates, freshwater algae, and phytoplankton are shown by the thatched bars in Figure 9 (35).

Chapman (36) used three different levels of water hardness for acute tests with Daphnia magna. The results showed that daphnids were three times more sensitive to lead in soft water than in hard water. The toxic levels were approximately 1 mg/ℓ. Several tests (37,38) showed that lead is acutely toxic to rainbow trout in the range of 1,000 to 500,000 µg/ℓ. The wide range is attributed to complexation of the lead by inorganic anions in hard waters. Chronic tests showed that most of the trout developed spinal deformities in hard water with concentrations of measured lead of 850 µg/ℓ and above, and in soft water with lead concentrations as low as 30 µg/ℓ (37). Similar to the trout data, Tarzuell and Henderson (39) and Pickering and Henderson (40) showed toxicity of lead with LC_{50} values from 2.4 to 542 mg/ℓ to fathead minnows depends upon the hardness of the water.

Monahan (41) and Malanchuk and Gruendling (42) reported acute effects to freshwater algae of from 500 to 26,000 µg/ℓ. Hessler (43) showed phytoplankton to be equally sensitive with inhibition and death is in the 2,000 to 60,000 µg/ℓ range.

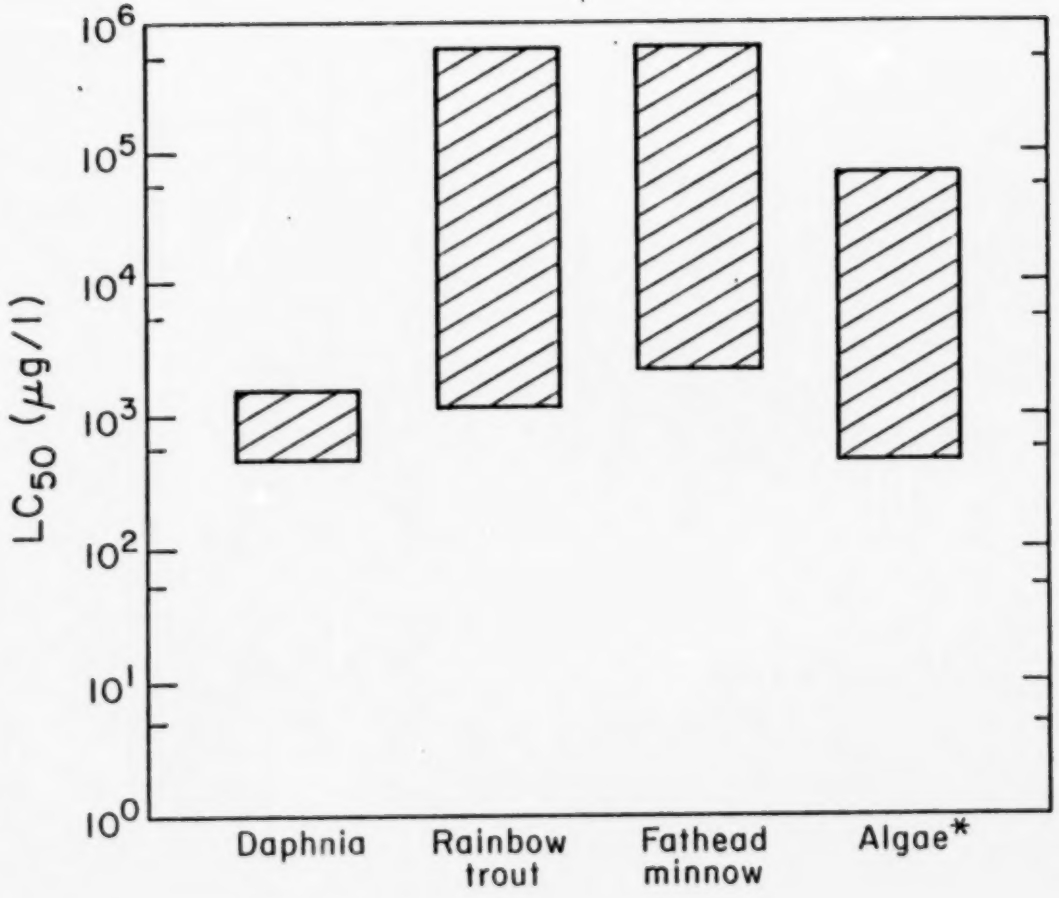

Figure 9. Toxicity Ranges of Lead to Various Bioassay Organisms (*EC_{50} for Algae)

CADMIUM

In general, the range of acute toxicity for cadmium to the bioassay organisms was about 10 to 100,000 µg/ℓ (44) (Figure 10). A reduction in the toxicity associated with increased hardness became evident for both the fish and invertebrate species.

Rainbow trout exhibited acute ranges of 96-hr LC_{50}'s in both static and flow-through tests of 1 to 7 µg/ℓ (38,46-48). A 200-hr LC_{50} value of 0.7 µg/ℓ for rainbow trout was determined by Chapman (45), not significantly lower than the 96-hr LC_{50} values.

After trout, Daphnia magna was found to be the next most sensitive species tested with LC_{50} values from 10 to 140 µg/ℓ (49,50). Fathead minnows were relatively insensitive with LC_{50}'s from 2,000 to 74,000 µg/ℓ (51,52).

Reduction in growth rate was the major toxic effect observed with aquatic algae. EC_{50} values for freshwater algae ranged from 5 to 250 µg/ℓ (44). Two species of saltwater phytoplankton had 96-hr EC_{50} values of 160 and 175 µg/ℓ based on growth inhibition (53). the values are considerably above the chronic values for other saltwater animal species tested (44).

COPPER

Copper toxicity is strongly dependent upon organic and inorganic complexation and, as such, varies widely with chemical characteristics of aqueous solutions (54). The range of copper toxicity for various bioassay organisms ranged from 5 to 8,000 µg/ℓ as shown in Figure 11 (55). Low alkalinity waters consistently showed higher toxicity.

Cairns, et al. (56) indicated that daphnids are more resistance to copper at low temperatures in acute tests. The LC_{50} for Daphnia magna ranged from 10 to 200 µg/ℓ (54). Rainbow trout are about equally sensitive as Daphnia with a range of LC_{50}'s of 20 to 500 µg/ℓ depending on the hardness of the water (45,57). Chakoumakos, et al. (57) have reported adult rainbow trout are approximately 2.5 to 3.0 times more resistance to copper than juveniles. Brown, et al. (58) showed a quantitative decrease in the acute toxicity of copper to trout with increases in naturally occurring organic chelating agents. The fathead minnow was about equally sensitive to copper as rainbow trout as shown in Figure 10 (55).

Copper is known for its algicidal and herbicidal characteristics. Concentrations that inhibit growth range from 1 to 8,000 µg/ℓ (55). Saltwater algae appear to be as sensitive as freshwater algae.

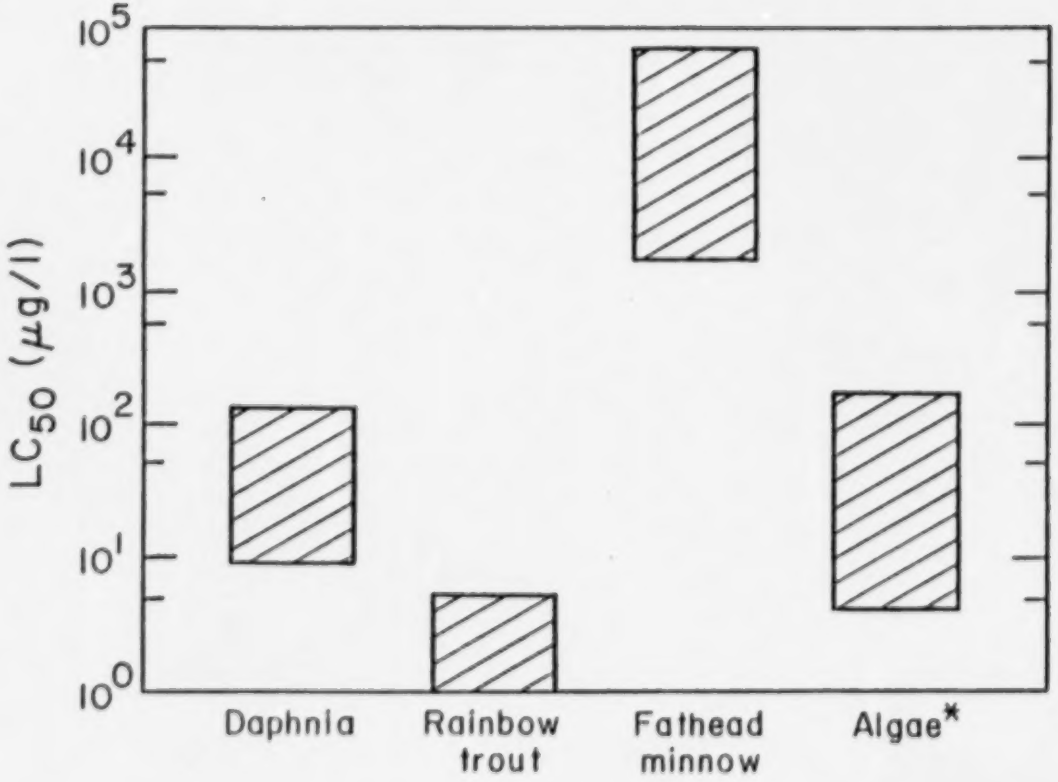

Figure 10. Toxicity Ranges of Cadmium to Various Bioassay Organisms (*EC_{50} for Algae)

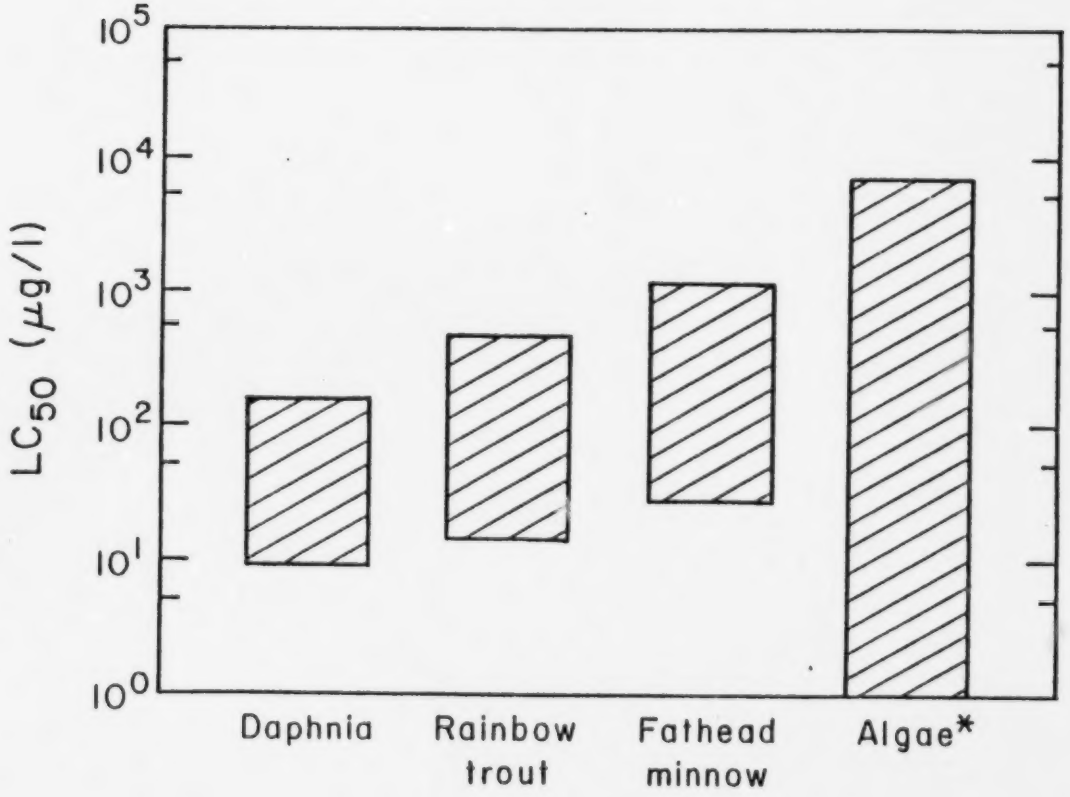

Figure 11. Toxicity Ranges of Copper to Various Bioassay Organisms (*EC_{50} for Algae)

ZINC

Zinc is an essential trace element for all organisms which, at higher concentrations, can be toxic. The range of acute values for freshwater organisms is from 30 to 38,000 µg/ℓ (Figure 12) and has been found to be similar for fish and invertebrates (59). The toxicity of zinc compounds to aquatic organisms is modified by several environmental factors, in particular, water hardness, dissolved oxygen and temperature. The EPA criterion for zinc in domestic water supplies is less than 5,000 µg/ℓ (60).

The LC_{50} value for Daphnia ranged from 100 to 655 µg/ℓ depending on the water hardness (36,61,62). Rainbow trout and fathead minnows showed much wider ranges of 90 to 4,700 µg/ℓ and 600 to 35,500 µg/ℓ, respectively (59). Algae exhibited growth inhibition effects from 30 to 5,100 µg/ℓ (59).

SILVER

Acute and chronic data for the toxicity of silver to aquatic organisms shows a wide variation. For the four bioassay groups in Figure 13, the acute values for silver range from 0.25 µg/ℓ for the Daphnia magna to 1,000 µg/ℓ for algae (63). The data base showed that the acute toxicity of silver apparently decreases as hardness increases. Daphnia exhibited an LC_{50} sensitivity range of 0.25 to 49 µg/ℓ with most values being in the few microgram per liter range (64). In comparison, rainbow trout data ranged from 5 to 280 µg/ℓ and fathead minnows from 4 to 230 µg/ℓ (64). Various growth inhibitions to algae occurred in the range of 30 to 1,000 µg/ℓ with complete inhibition of growth at a few hundred micrograms per liter (63).

HEPTACHLOR

Heptachlor has been a widely used pesticide for general insect control throughout the United States. It is a chlorinated hydrocarbon which has been shown to be toxic to aquatic life, to accumulate in plant and animal tissues and to remain in aquatic ecosystems (65). In general, LC_{50} values range from about 10 to 1,000 µg/ℓ as shown in Figure 14. The data on acute toxicity for static testing in Figure 13 is based on initial dosed levels, not exposure concentrations. All values in Figure 14 are expressed as technical grade heptachlor.

The LC_{50} value for Daphnia was reported as 78 µg/ℓ (66). Rainbow trout exhibited LC_{50} values of 10 to 27 µg/ℓ (67,68) and fathead minnows, 78 to 130 µg/ℓ (69). EC_{50} values for freshwater algae were determined as 27 to 39 µg/ℓ and for saltwater species, 93 to 2,260 µg/ℓ (65).

In general, toxicity increased with time of exposure. The effect of increased exposure time on LC_{50} values showed greater increases for the invertebrates than for the vertebrates.

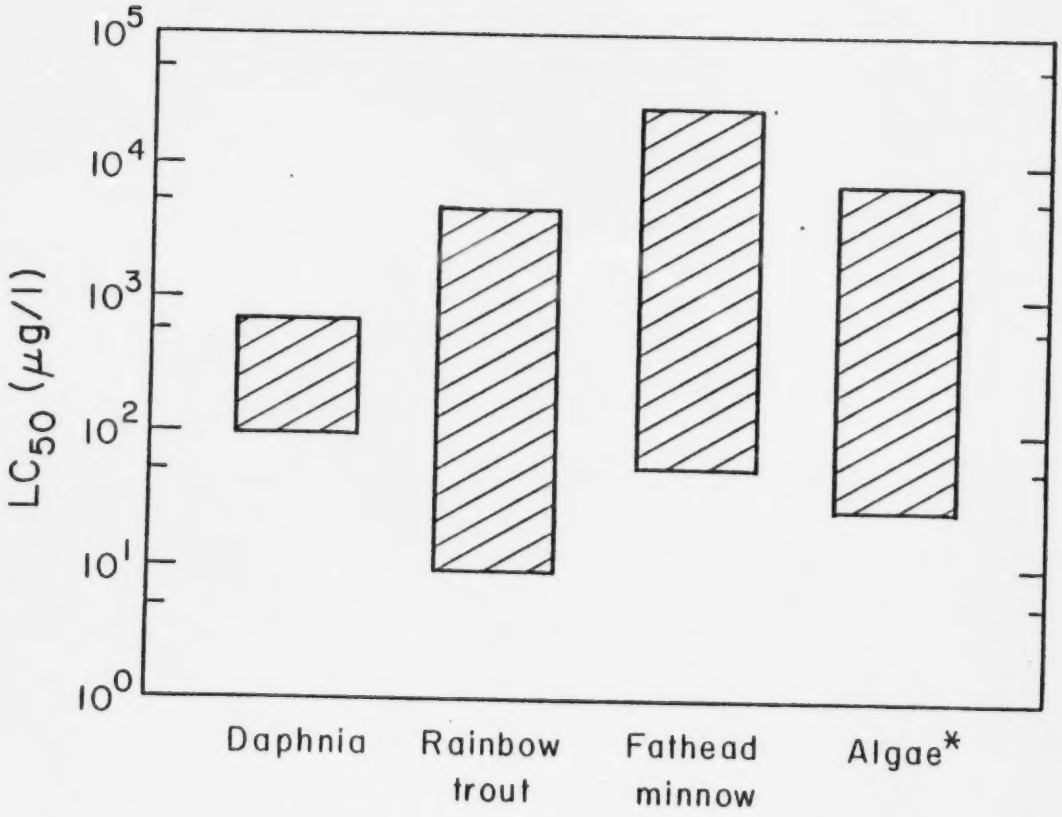

Figure 12. Toxicity Ranges of Zinc to Various Bioassay Organisms (*EC_{50} for Algae)

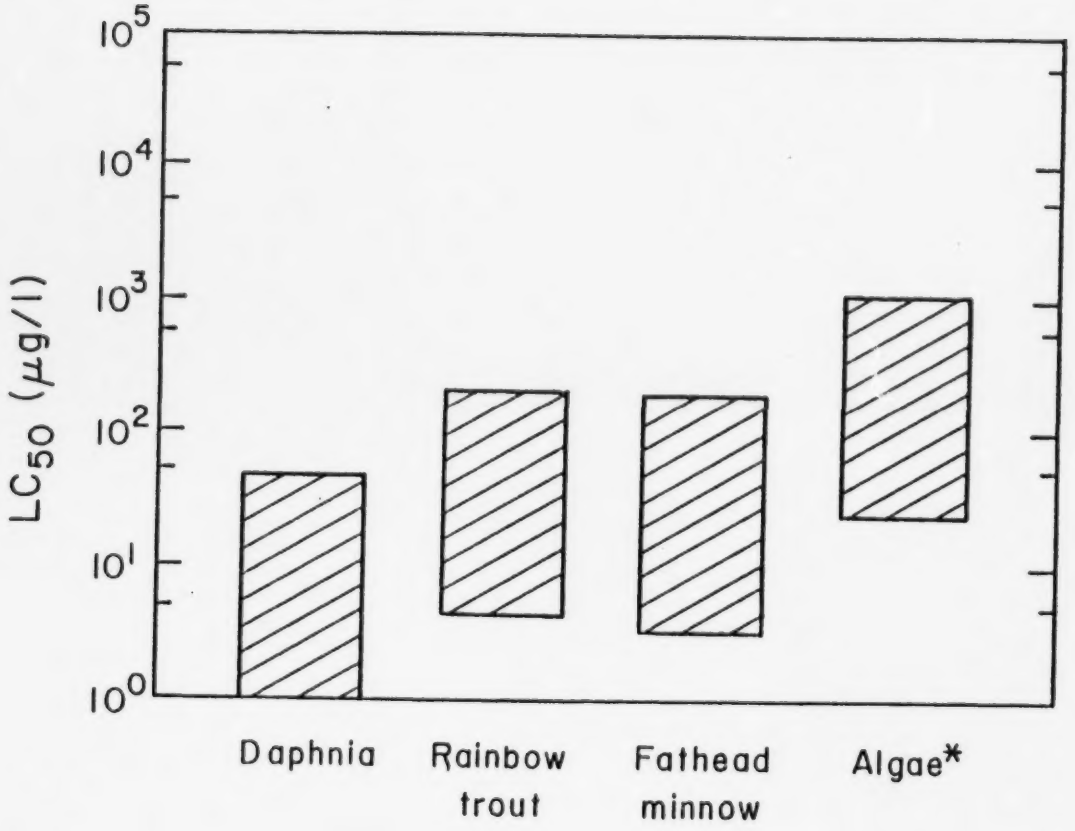

Figure 13. Toxicity Ranges of Silver to Various Bioassay Organisms (*EC_{50} for Algae)

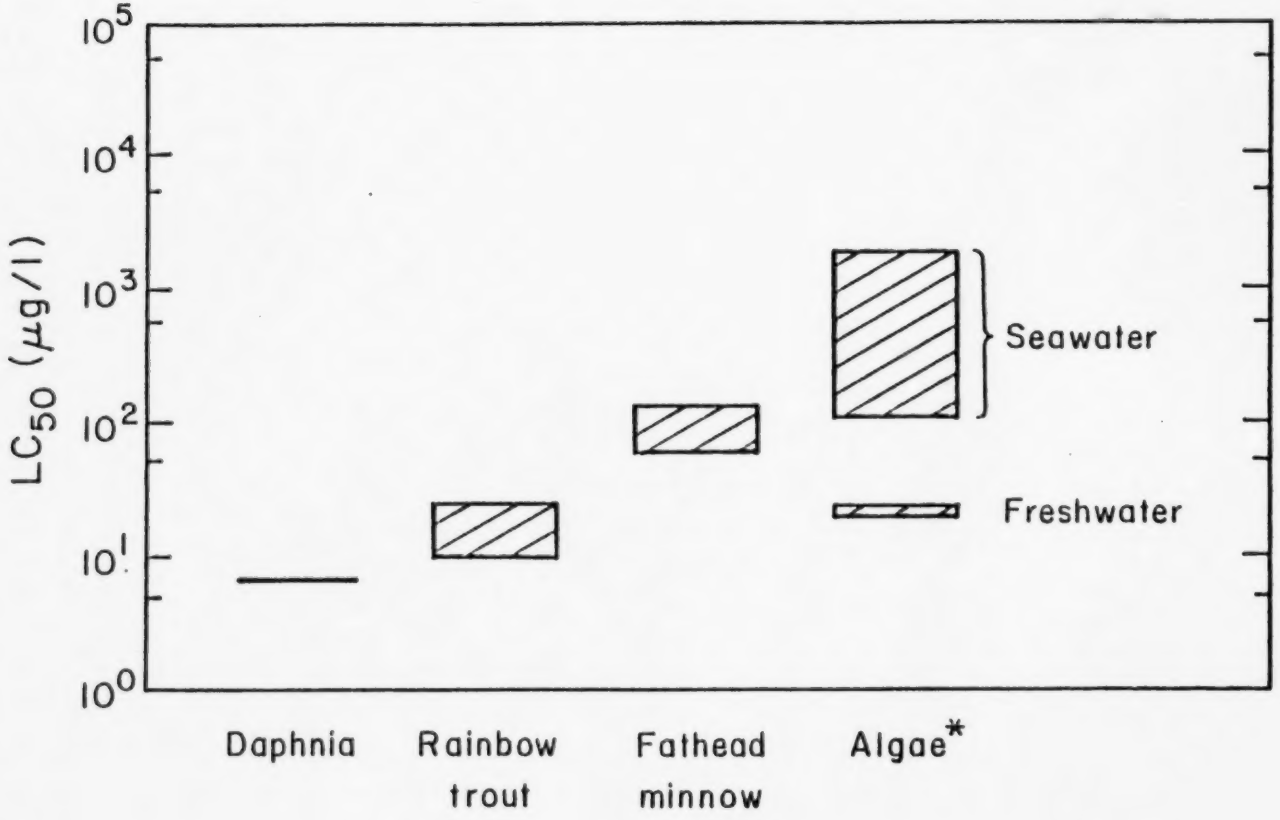

Figure 14. Toxicity Ranges of Heptachlor to Various Bioassay Organisms (*EC_{50} for Algae)

ENDOSULFAN

Endosulfan is an insecticide developed in the mid-fifties for insect control on vegetables, fruits and tobacco. Currently it is on the EPA restricted list limiting its current use (70). Either technical grade endosulfan or mixtures containing such were used for most of the toxicity testing.

The vertebrate (fish) species tested were shown to be more sensitive than the invertebrates tested (Figure 15) with LC_{50} values for trout and fathead minnows in the low µg/ℓ range and Daphnia in the 100's of µg/ℓ range (70). Little data were available for either freshwater or saltwater algae.

Pickering and Henderson (71) studied the effect of water hardness on toxicity of endosulfan and observed no significant effect. It was generally shown that the toxicity of endosulfan increased with increasing temperature (70). Significant differences were noted between nominal and measured exposure concentrations (72).

PARATHION

The toxicity of parathion, an organophosphorus pesticide, ranged from less than one µg/ℓ for sensitive species like Daphnia to about several µg/ℓ for more resistance species like fathead minnows (Figure 16). Space (73) found the three-week LC_{50} for Daphnia to be 0.14 µg/ℓ and the 96-hr LC_{50} in flow-through bioassays to be 0.62 µg/ℓ. LC_{50} values were about 1.8 µg/ℓ for trout and from 1.6 µg/ℓ for fathead minnows. No data were available for algae.

COMPARISON OF NITRIFYING BIOASSAY TO OTHER TEST ORGANISMS

For the Nitrobacter bioassays the ranges shown in Figure 5 can be compared directly with the LC_{50} values shown in Figures 9 through 16. Such comparisons are shown in Table 4. These comparisons show that in general Nitrobacter exhibits comparable sensitivity to the metal toxicants, but little sensitivity to the toxic organics. This would be expected since the toxic organics operate through specific enzyme mechanisms that are absent in bacteria.

For the Nitrosomonas bioassays, the ranges shown in Figure 3 can be compared directly with the LC_{50} values shown in Figures 9 through 16. Such comparisons are shown in Table 5. These comparisons show that in general Nitrosomonas exhibited a range from low to high sensitivity for the metal toxicants, but less sensitivity to the toxic organics. The reduced sensitivity for the organics compared to the other bioassay organism would be expected as stated above. In general, Nitrosomonas showed less sensitivity than Nitrobacter.

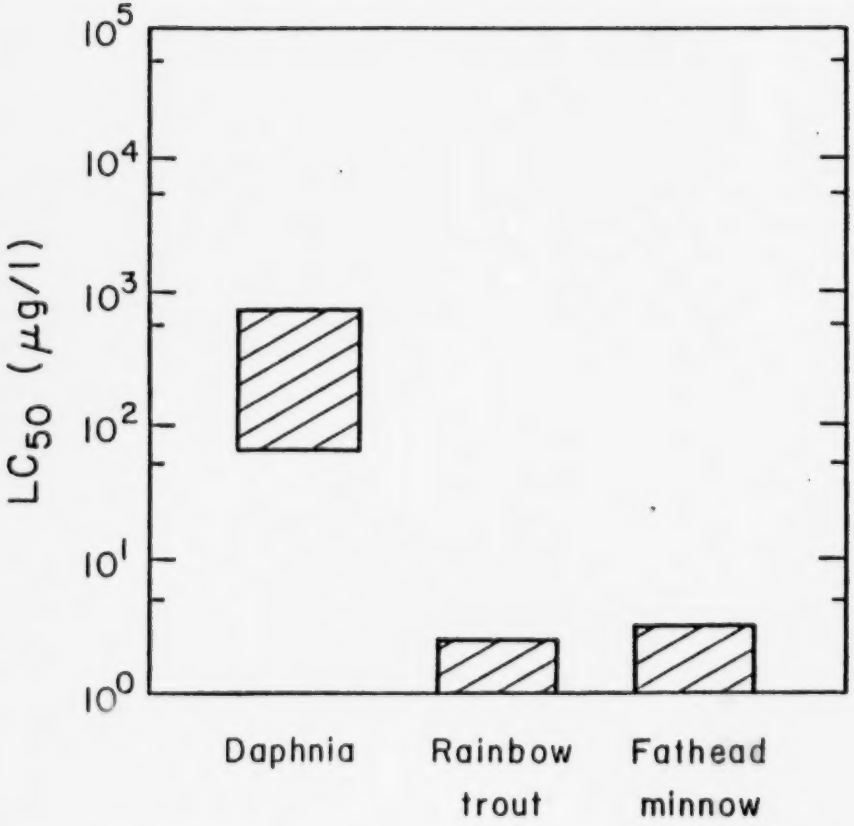

Figure 15. Toxicity Ranges of Endosulfan to Various Bioassay Organisms.

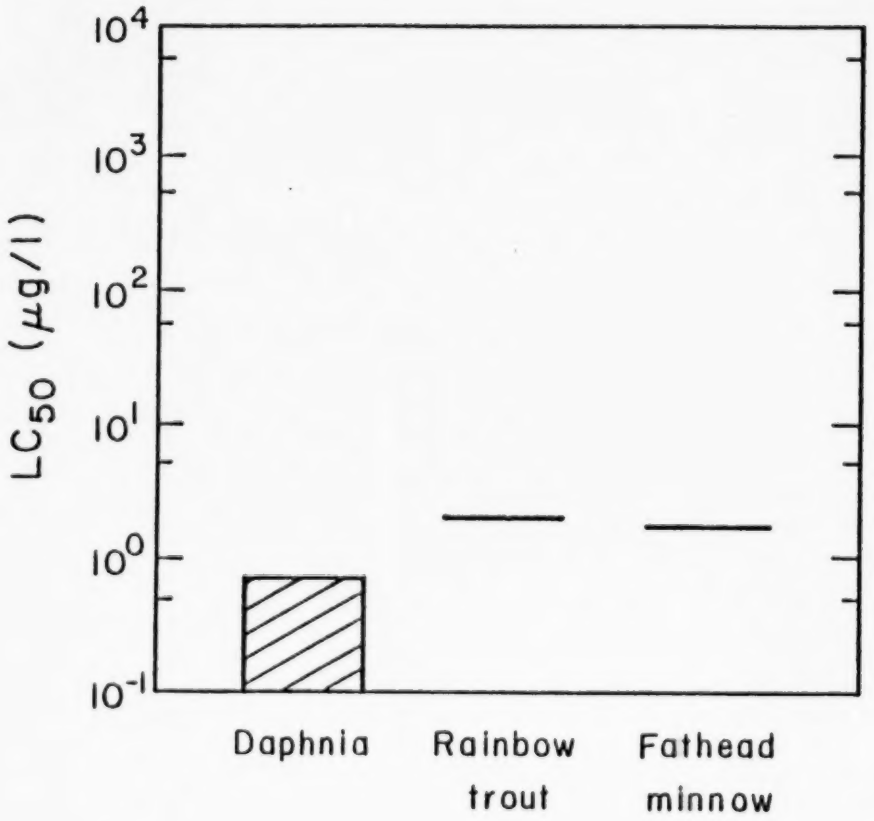

Figure 16. Toxicity Ranges of Parathion to Various Bioassay Organisms.

TABLE 4. COMPARISON OF SENSITIVITY OF *NITROBACTER* TO OTHER BIOASSAY ORGANISMS*

Toxicant	Bioassay Organism			
	Daphnia	Trout	Fathead Minnow	Algae
Lead	0	+	+	0
Cadmium	-	-	0	-
Copper	-	-	-	0
Silver	+	0	+	+
Zinc	-	-	-	-
Endosulfan	-	-	-	x
Heptachlor	-	-	-	-
Parathion	-	-	-	-

* +, more sensitivity; 0, comparable sensitivity; -, less sensitivity; x, no data available

TABLE 5. COMPARISON OF SENSITIVITY OF *NITROSOMONAS* TO OTHER BIOASSAY ORGANISMS*

Toxicant	Bioassay Organism			
	Daphnia	Trout	Fathead Minnow	Algae
Lead	-	+	+	0
Cadmium	-	-	0	-
Copper	-	-	-	-
Silver	+	+	+	+
Zinc	-	0	0	0
Endosulfan	-	-	-	x
Heptachlor	x	-	-	-
Parathion	-	-	-	x

* +, more sensitivity; 0, comparable sensitivity; -, less sensitivity; x, no data available

SECTION 8

BACTERIAL BIOASSAY APPLICATION TO INDUSTRIAL WASTEWATER TREATMENT

Toxic industrial wastes threaten both biological waste treatment systems and the environment of their ultimate disposal. Both of these problems can be addressed through the use of Level 1 bioassays. In this section the use of Nitrobacter bioassay is described for evaluation of industrial waste toxicity to biological wastewater treatment, specifically activated sludge.

METHODS OF MEASURING TOXICITY TO ACTIVATED SLUDGE

Many different bioassay methods have been used to measure a toxic response in activated sludge. In this section several of these methods will be described and their advantages and disadvantages summarized.

The most common method is to use continuously fed reactors. Duplicate reactors are fed with and without the toxicant and the organic removal rate is monitored. This technique is expensive to operate and extremely time consuming. Equipment requirements include reactors and constant-feed pumps, and a large source of industrial waste is necessary. This method does approximate the conditions in activated sludge tanks, although the hydraulic and solids retention times of the activated sludge treatment are difficult to maintain. Short-term studies often never approach assumed steady-states and long-term studies do not provide information rapidly enough.

Unfortunately, continuously fed reactors are notoriously difficult to run. Pumping small amounts of influent at a controlled rate is difficult. Several weeks may be necessary for stabilization of growth to occur. Often the effects of toxicity are masked by the normal variability in COD or BOD removal. For these reasons, continuously fed reactors cannot be used as a standard analysis procedure. Examples of toxicity tests performed by this method are reported by Moulton and Shumate (74), Ayers, Shumate, and Hanna (75), and Barth, et al. (76).

Another common toxicity test is the BOD bottle technique. There are many variations, but this technique essentially consists of measuring the BOD of a known organic with or without the toxic waste. The method is extremely simple and can be accomplished in standard treatment plant laboratories. However, the dilution of the waste for the test may reduce its concentration below its threshold limit value. In addition, the small range of only 2 to 7 mg BOD/ℓ greatly limits its accuracy. Mowat (77) used this method to determine the toxicity of various metals to mixed heterotrophic growth from sewage.

Most of the difficulties of the BOD bottle technique can be alleviated by using a batch-fed technique. This technique involves measurement of the substrate utilization rate and the cell mass. The most common method of determining the substrate utilization rate is manometrically as described by Hartmann and Laubenberger (15), although the decrease in concentration of the organics can also be used as described by Bunch and Chambers (78). The cell mass can be measured as total suspended solids, volatile suspended solids (79), organic nitrogen (15), or ATP (80).

Batch-fed techniques typically require more advanced instrumentation than available at industrial treatment plants. Respirometers are expensive and difficult to operate even for highly trained personnel. Specific measures of organics will require sophisticated instruments or difficult chemical extractions. The length of the tests using batch-fed techniques are usually long compared to the generation times of the heterotrophs, so the number and types of organisms can vary significantly between the reactors with toxicants and the control.

None of the three methods (continuously fed, BOD bottle, batch-fed) are directly applicable for determining the toxicity of industrial wastes by plant personnel. These tests primarily require too much effort or advanced instrumentation. The assessment of toxicity by the use of the Nitrobacter bioassay is shown below.

LABORATORY STUDIES WITH KNOWN TOXICANTS

To determine the ability of the Nitrobacter bioassay to predict toxicity to biological waste treatment, laboratory studies were undertaken with two known toxicants. Three 4-liter activated sludge units with internal clarifiers were used as complete-mixed reactors. The units were fed primary clarifier effluent from the Corvallis Sewage Treatment Plant to provide a hydraulic detention time of 18 hours; the solids retention time was not controlled, but cells were wasted daily to provide a food to microorganism ratio of approximately 0.2 mg BOD/mg TSS-day. Treatment efficiencies were measured as COD removal across the reactors.

After 4 days of operation, 50 mg/ℓ of zinc (as $ZnSO_4$) was fed with the sewage to one of the reactors, and 10 mg/ℓ of trichlorophenol (TCP) was fed with the sewage to the second reactor. The third reactor was operated as a control.

The results are shown in Figure 17a. The reactor dosed with zinc exhibited a dramatic change of floc characteristics within 24 hours; the floc became more disperse and lighter in color than the control. The floc dosed with TCP became darker in color than the control. Both units showed decreased COD removal as compared to the control which clearly indicated a toxic response.

On the twelfth day of the tests the Nitrobacter bioassay was conducted on the two toxic feeds and the control feed. The relative metabolism rates

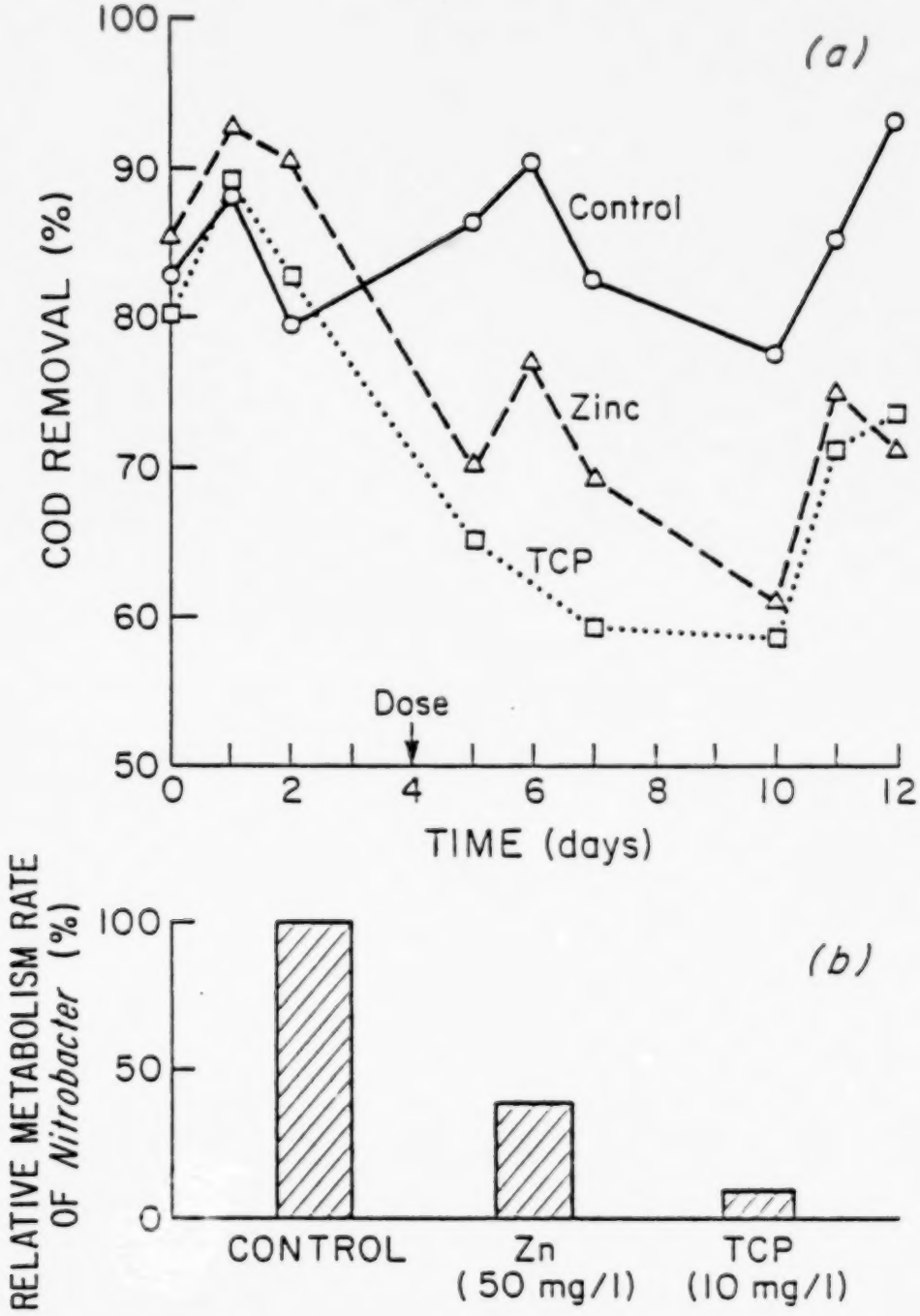

Figure 17. COD Efficiency in Response to Zn, TCP Toxicity (a) and Relative Metabolism Rate of Nitrobacter at 50 mg/ℓ Zn and 10 mg/ℓ TCP (b).

of Nitrobacter for the 50 mg/ℓ zinc and 10 mg/ℓ TCP with respect to the control were 40 percent and 16 percent, respectively (Figure 17b).

These results show that the Nitrobacter bioassay test had adequate sensitivity to detect these two toxic compounds at levels that produced measurable toxicity to activated sludge.

LABORATORY STUDIES WITH A METAL PROCESSING WASTEWATER

Another series of tests using bench-scale activated sludge reactors were conducted as described above except a toxic industrial waste was mixed with the feed at 0 percent (control), 10 percent and 20 percent. The wastewater sampled was from an industry that processes various metals and was known to contain a wide range of organics, chlorinated organics, and metals.

The concentrations of 10 percent and 20 percent of the industrial waste caused no visible change in the activated sludge floc. However, the COD efficiencies were significantly lower than the control without the wastewater addition for the 20 percent industrial waste feed (Figure 18a).

Seven days after dosing the activated sludge units with the industrial waste, the Nitrobacter bioassay was run one each feed. The relative rate of Nitrobacter metabolism for the feeds with 10 percent and 20 percent industrial waste were 50 percent and 4 percent, respectively.

These results showed that the Nitrobacter bioassay could detect unspecified toxicity in industrial wastewaters. As such, the Nitrobacter test could be specifically applied as a Level 1 bioassay in the identification of toxic wastestreams and appropriate treatment and control technologies.

FIELD STUDIES WITH A FIBERBOARD MILL WASTEWATER

The Nitrobacter bioassay was applied to an industrial wastewater treatment problem at a fiberboard manufacturing site. The manufacturing process at this mill is known as the "wet process" (Figure 19). Wood chips and scrap from a neighboring lumber mill are ground into small particles. The solids then are treated with high pressure steam that removes the water soluble material from the wood leaving a fibrous skeleton. The fibers then are treated with a preservative and bonding resin, and mixed with reuse water to form a slurry (0.5 percent solids by weight). The slurry is poured onto a wire screen that carries the fibrous mat to a press where the board is finally formed.

Excess water removed during the manufacturing process is discharged to an on-site treatment facility consisting of settling ponds, a completely mixed activated sludge basin, and a small clarifier (Figure 20). The waste solids from the clarifier are land applied and the clarifier effluent is recycled to the plant as reuse water.

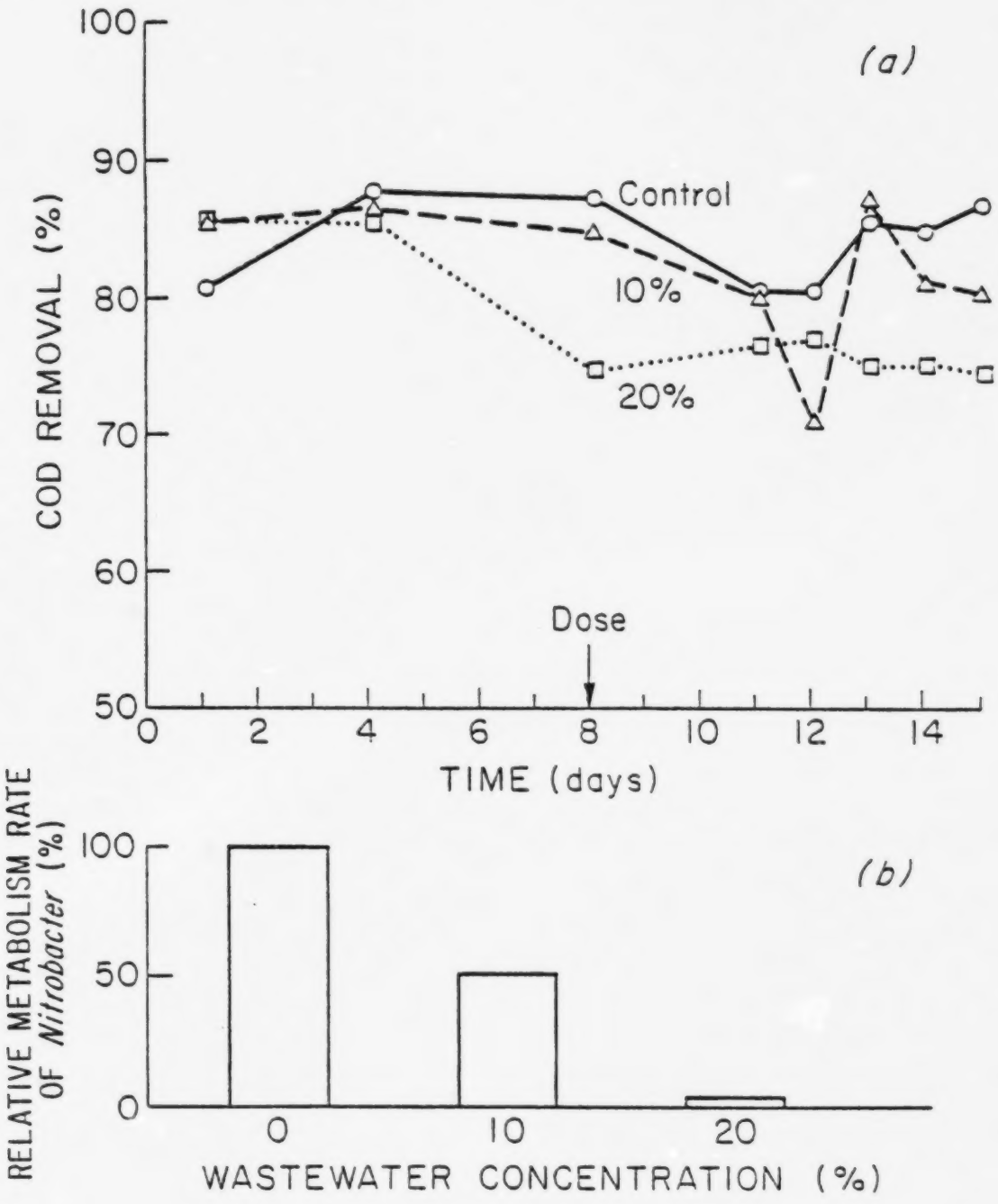

Figure 18. COD Efficiency in Response to a Toxic Industrial Wastewater (a) and Relative Metabolism Rate of Nitrobacter versus Wastewater Concentration (b).

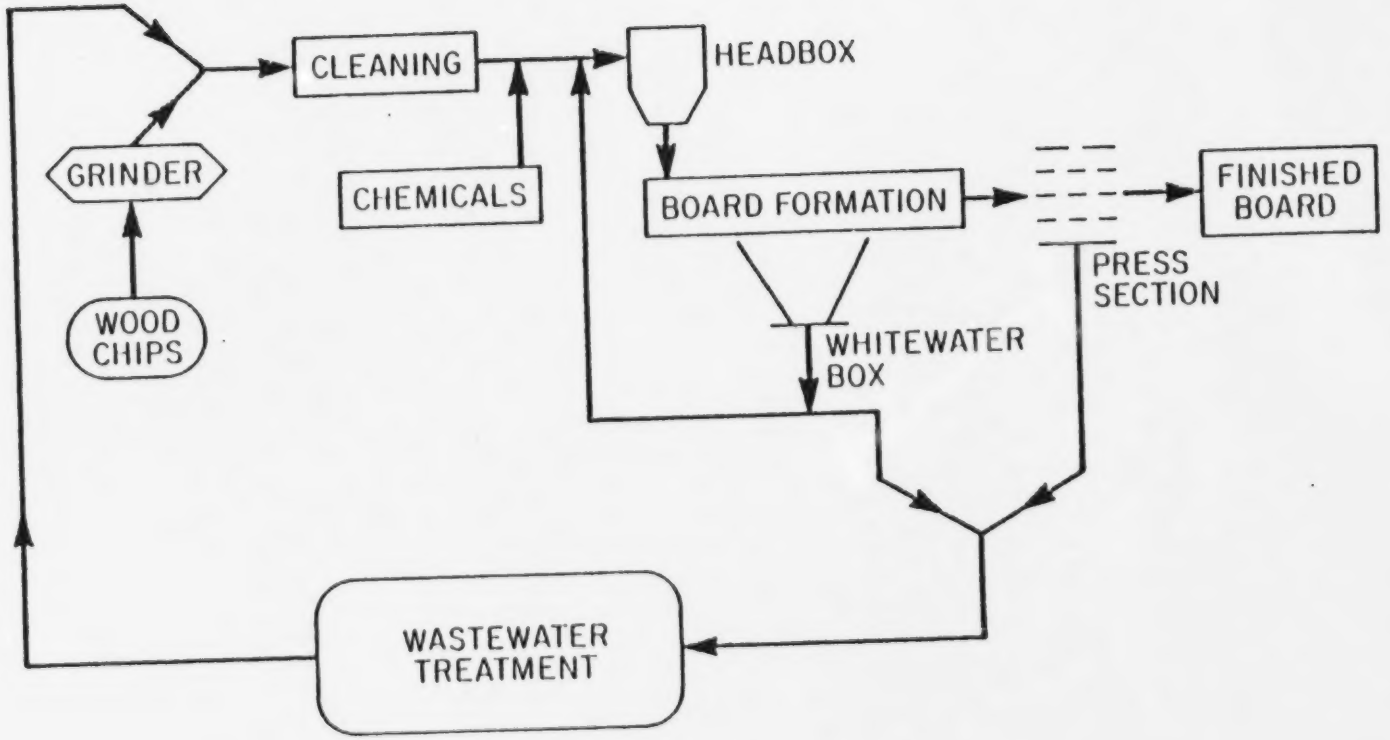

Figure 19. Flow Diagram for Fiberboard Manufacturing Operation.

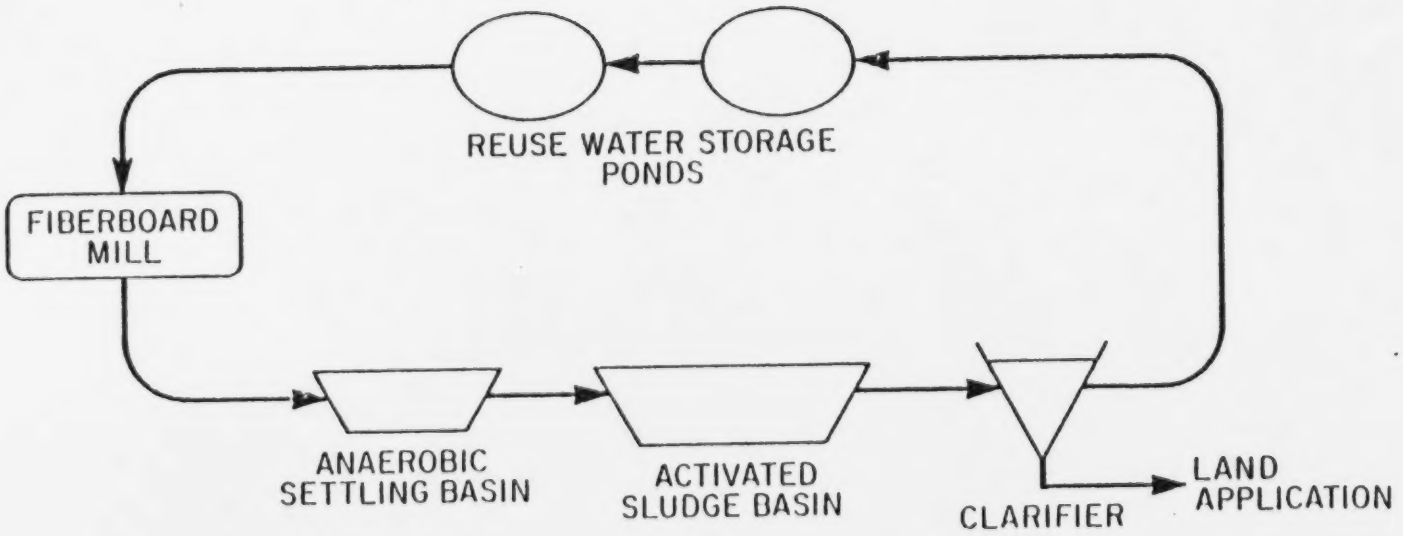

Figure 20. Flow Diagram for Fiberboard Wastewater Treatment Process.

The mill wastewater is pumped to a primary settling pond at an average flow rate of 3×10^5 ℓ/d. The wastewater temperature is 43°C with a pH of 4.5. The settling pond has a volume of 1.5×10^6 ℓ and a hydraulic detention time of about 5 days.

The aeration basin has a 1.5×10^6 ℓ capacity and a 5 day hydraulic detention time. Aeration is supplied by five surface aerators that maintain 4 to 6 mg/ℓ of dissolved oxygen. Ammonium nitrate and diammonium phosphate are added as nutrients. The pH is raised to 6.8 by biological activity. The clarifier holds 4×10^4 ℓ with a hydraulic dentention time of about 1.5 hours.

The main component of the wastewater are the wood sugars that results from the steaming process. Some of the wood used for mill feed has been treated by the supplier with a fungicide known to contain pentachlorophenol. Phenyl formaldehyde is added to the cleaned wood chips as a preservative; alumina, a "waxy emulsion" and "defoamer" are added to aid in the board formation. No slimicides are added during the process, although slime appears to have been a problem in the past (81).

In 1971, this company developed a completely closed wastewater system to minimize any possible stream pollution. Treatment efficiency of the activated sludge unit has dropped considerably from 1975 to 1978. BOD removal has been reduced to nearly 50 percent, while the suspended solids of the system had increased to over 6000 mg/ℓ. The Nitrobacter bioassay was performed on the activated sludge influent and effluent as a Level 1 phase of investigation. A 10 percent solution of the activated sludge influent showed a 10 percent relative metabolism rate of the Nitrobacter which indicated significant toxicity (Figure 21). A 10 percent solution of the activated sludge effluent showed a 40 percent relative metabolism rate of Nitrobacter. This indicated that the toxicity was not being significantly removed by the biological waste treatment.

In an attempt to pinpoint the source of the toxicity, additional samples were taken at various point sources along the production line. No particular toxic discharge sites were isolated. A general increase of toxicity in the wastewater seemed to occur during the process with the toxicant being recycled through the wastewater treatment system.

Several physiochemical wastewater treatment methods were applied to the mill effluent in an effort to identify the optimal method to remove the toxicity. These include filtration with glass fiber filters, dissolved air flotation at 40 psig pressurization, followed by 10 minutes of mixing and 20 minutes of flotation and powdered activated carbon addition at 50 mg/ℓ. A 20 percent solution of the mill effluent was found to exhibit a 63 percent relative metabolism rate of Nitrobacter. Treatment of this 20 percent solution with either filtration or dissolved air flotation showed only a small increase in the relative metabolism rate of Nitrobacter, while powdered activated carbon removed almost all of the observed toxicity (Figure 22).

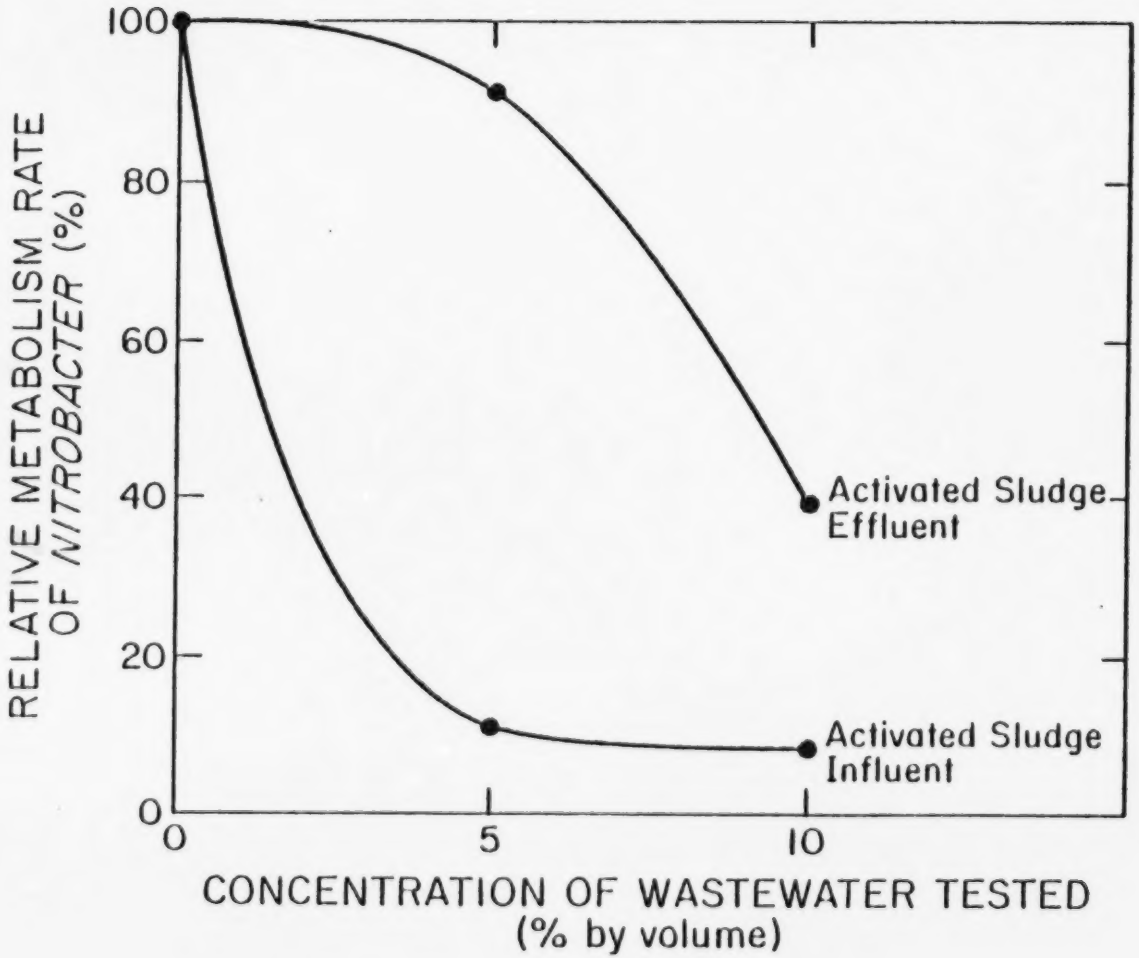

Figure 21. Relative Metabolism Rate of Nitrobacter versus Concentration of Wastewater Tested.

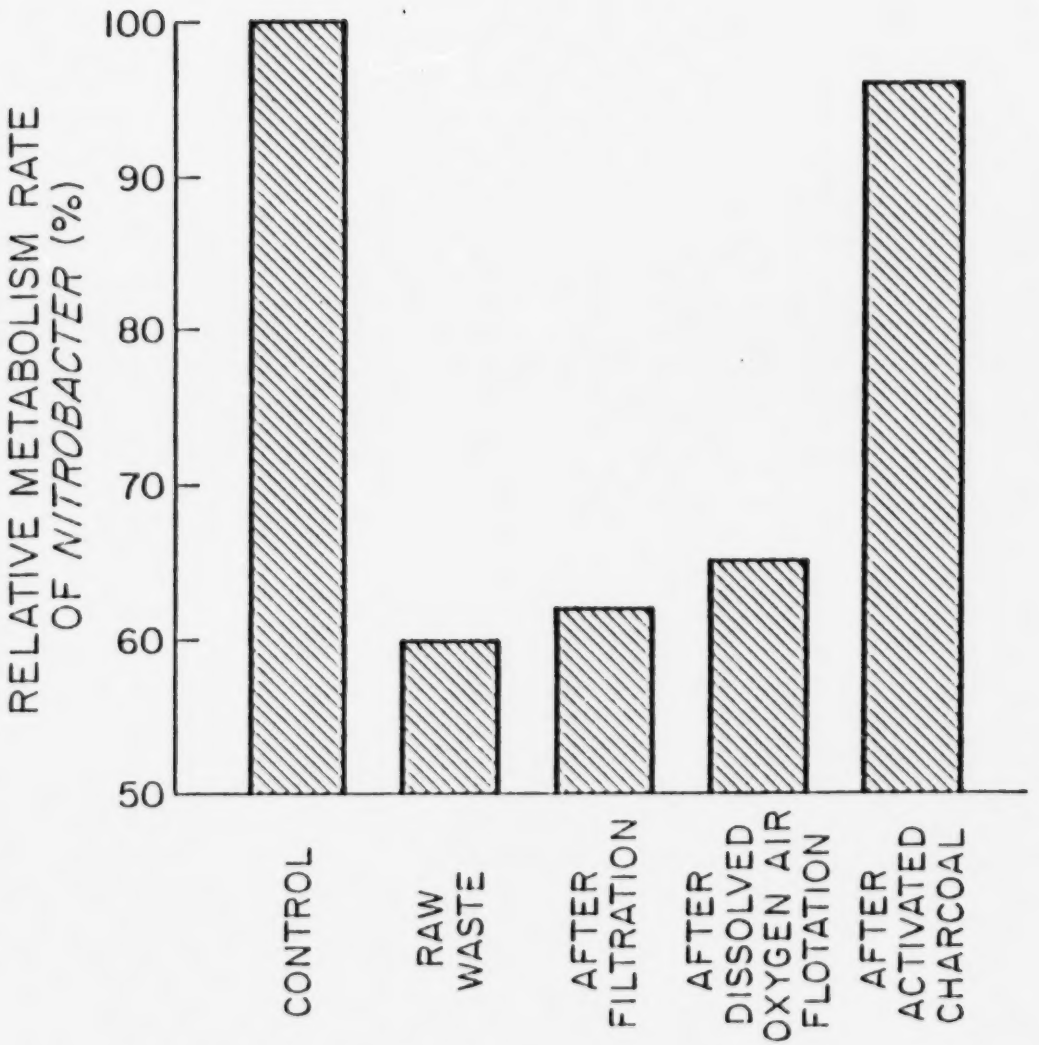

Figure 22. Evaluation of Several Wastewater Treatment Methods Using the Nitrobacter Bioassay.

Level 1 analysis using the Nitrobacter bioassay identified the wastewater from this plant as being toxic to the activated sludge operation. To date, this problem has caused no noticeable deterioration in the quality of the finished fiberboard; however, the continued failure of the treatment system has caused company officials some concern with the assurance of continued quality. A Level 2 analysis was conducted to identify possible causes for the toxicity observed. Pentachlorophenol, used as a preservative by the wood chip supplier, was identified in the plant effluent at levels of 2 to 3 ppb. Pentachlorophenol is known to be toxic to bacteria by uncoupling oxidative phosphorylation and also to denaturing proteins. The current literature values for acute toxicity levels for pentachlorophenol are listed as 25.0 ppb for invertebrates, 14.0 ppb for freshwater fish and 7.5 ppb for algae (82). Although it is not believed that pentachlorophenol accounts for all of the observed toxicity, it is considered to contribute to the toxicity problem. A Nitrobacter bioassay verified fungicide toxicity (Figure 23). Other possible wastewater constituents known to be present that might exhibit a toxic effect are 2,4,6-trichlorophenol, phenyl formaldehyde, and resin acids.

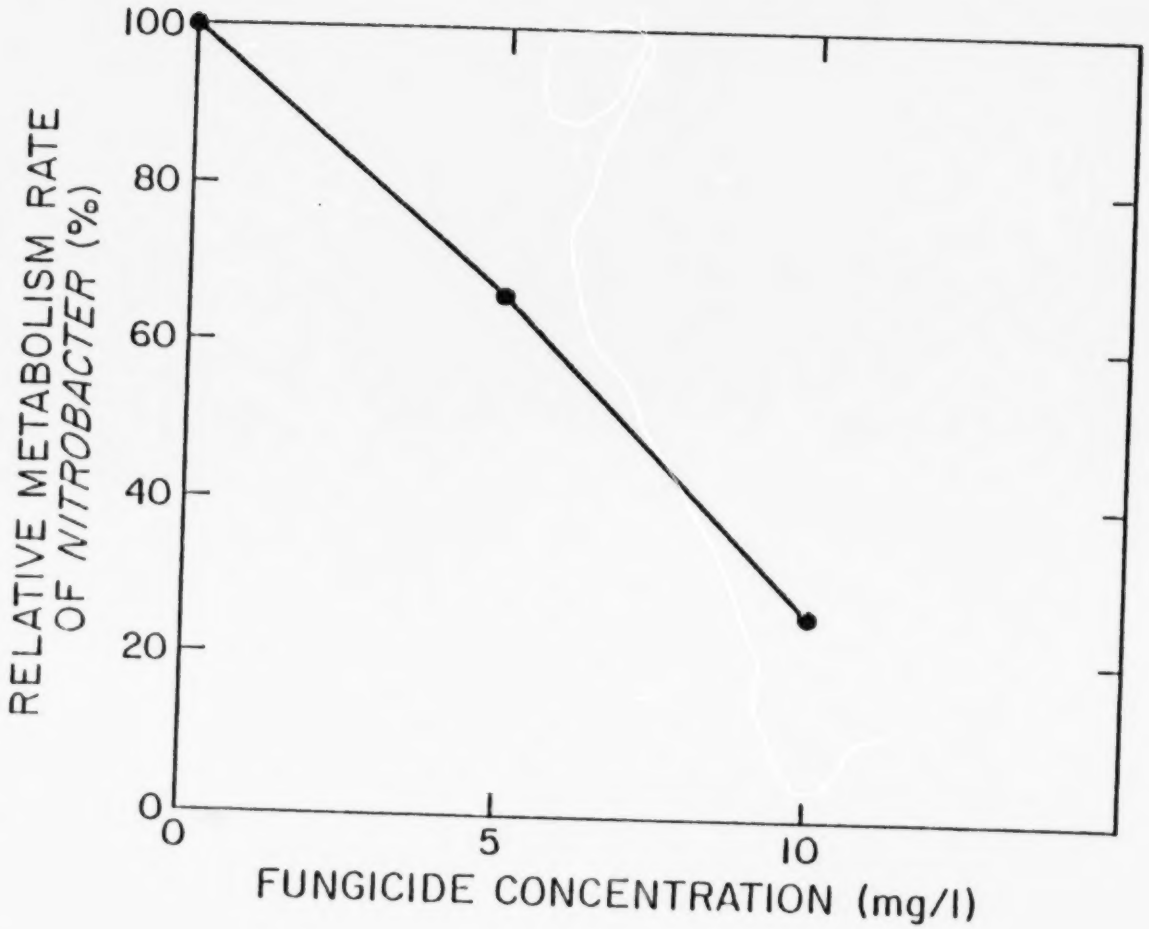

Figure 23. Assessment of Fungicide Toxicity using the Nitrobacter Bioassay.

SECTION 9

BACTERIAL BIOASSAY APPLICATION TO SOLID WASTE LEACHATE

In the Resource Conservation and Recovery Act of 1976, Congress directed the Environmental Protection Agency (EPA) to develop a regulatory program to manage and control the country's hazardous solid wastes from generation to final disposal. The EPA has formulated rules to implement this program (105), including identification and listing of hazardous wastes, and containing criteria for determining whether a waste is hazardous. Consequently, the EPA has proposed the Extraction Procedure (EP) for toxicity evaluation; this procedure is a specified 24-hour extraction of the solid waste using acetic acid. No bioassay evaluation is required.

The goal of the EP is to model the fate of a poorly managed hazardous waste; that is, a toxic waste that is disposed of in a municipal landfill or open dump located over an aquifer used for drinking water. The leachates generated by the EP are analyzed for the toxic components listed in Table 6 with their corresponding maximum permissible concentrations; these limits are 100 times the EPA National Interim Primary Drinking Water Standards (83).

Although the EPA rules do not require bioassay techniques in the evaluation of a waste for toxicity, their inclusion in the rules may occur in the future. Toward this end the EPA has conducted bioassay tests to evaluate the use of EP leachate in determining acute and chronic aquatic toxicity, phytotoxicity, and mutagenecity (830. Bioassay tests which have already been used in leachate toxicity tests include acute toxicity to Daphnia magna; radicle elongation studies using radish, sorghum, wheat, and soybean plants; and bacterial gene mutation, fungal microorganism mutation, and bacterial DNA repair studies (84). Leachates from a number of wastes have been studied, including municipal sewage sludges (85,86), textile wastes (87), fly ash (88,89), in situ coal gasification byproducts (90), flue gas desulfurization scrubber sludges (91), plastics wastes (92), radioactive wastes (93), zinc-carbon battery wastes (94), zinc smelting wastes (95), coal mining spoils (96), municipal landfill wastes (97,98,99,100), and steel and aluminum manufacturing wastes (101). The EPA has studied leachates from such wastes as soybean process cake, platers waste, retorted oil shale, dye waste, municipal sewage sludge, power generation ashes and sludges, and gasification wastes (102).

Many of the parameters in extraction procedures have been set at values considered to be typical of conditions found within municipal landfills. Small differences in such variables as complexing ligand concenrations and pH may cause a wide variation in the solubilities of metals. Theis, et al.

TABLE 6. MAXIMUM PERMITTED LEVELS OF TOXIC CONTAMINANTS

Contaminant	Concentration (mg/ℓ)
As	5.0
Ba	100.0
Cd	1.0
Cr	5.0
Pb	5.0
Hg	0.20
Se	1.0
Ag	5.0
Endrin	0.02
Lindane	0.40
Methyoxychlor	10.0
Toxaphene	0.50
2,4-D	10.0
2,4,5-TP (Silvex)	1.0

(88) found that pH was important in determining the solubilities of metals in fly ash, while Ham, et al. (103) found that increasing leachate acidity released higher quantities of inorganic ions from a number of industrial wastes. Other investigators have determined that pH, complexing ligand concentration, and adsorbing surface availability are important in determining the total solubility, speciation, and distribution of metals in solution (104-110).

The EP specifies acidification of a sludge suspension to pH 5.0 with acetic acid. The final acetate concentration may not exceed 0.1 M which may limit the final pH value to higher than this. Leachates from actual landfills have exceeded these limits. Chian and DeWalle (100) found a pH range of 3.7 to 8.5 and a range of volatile acids concentrations from 0.03 M to 0.3 M in municipal landfill leachates, and Burrows and Rowe (97) found up to 0.8 M volatile acids in municipal landfill leachates. Leachates from landfills are also known to contain such substances as humic or fulvic acids and amino acids which can act as powerful complexing agents and increase the total solubilities of metals in the leachates (99). The EP does not include complexing ligands other than acetate, which forms only relatively weak complexes with most metals.

In this section the use of the Nitrobacter bioassay is described for evaluation of the leachate from the EPA extraction procedure for a solid waste. As part of additional studies, the chemical composition of the extractant was varied to determine its effects of the chemical characteristics of the leachate; as such, bioassays were conducted on leachates extracted with distilled water, EDTA, and acetate. A basic oxygen furnace scrubber sludge was chosen as the solid waste.

BOF SCRUBBER SLUDGE

Basic oxygen furnace (BOF) scrubber sludges are composed mainly of iron oxides, but also contain large amounts of calcium and magnesium from lime used for pH control. BOF scrubber sludges often contain significant quantities of heavy metals, including zinc, lead, chromium, cadmium, and manganese. These metals originate from the use of scrap metal in the BOF charge. A preliminary analysis of the sludge used in this research revealed elevated levels of zinc, lead, and manganese (see Table 7). The total solids concentration of the sludge was 72.4 percent and the alkalinity was 114 mg/g dry. The pH was 12.5.

EXTRACTION

The three leaching solutions used were distilled water (aqueous solution), 0.15 M acetate solution, and 0.0375 M (0.15 N) EDTA solution. Distilled water was used to study the effects of pH alone, acetate was chosen to study the effects of a mild complexing ligand commonly found in municipal landfill leachates, and EDTA was chosen to study the effect of a very strong complexing ligand.

TABLE 7. SLUDGE METALS ANALYSIS

Metal	Concentration in Sludge, µg/g dry wt.	Maximum Solubility* mg/ℓ
Ca	21685	1570
MG	5428	393
Sr	15	1.1
B	285	20.6
Fe	98108	7100
Al	3094	224
Mn	2942	213
Zn	29420	2130
Cu	77	5.6
Cr	232	16.8
Ni	26	1.9
Cd	51	3.7
Pb	3108	225
As	26	1.9
Mo	552	40
Se	140	10
Zr	14	1
Sb	83	6
Co	28	2
V	28	2

*Based on 10 mℓ leachate per gram of sludge, the dilution factor in leaching tests.

Sludge was extracted by adding 10 mℓ of leaching solution for each gram of sludge. The solutions were shaken in a wrist-shaker for 24 hours.

BIOASSAYS

The bioassays were conducted as described in Section 5. Leachates were added at levels of 0 to 90 percent of the bioassay solution. The pH was varied by the addition of HCl from 5 to 8 but held constant throughout each test.

RESULTS

The relative activities obtained in each toxicity test were plotted for each leachate as shown in Figures 24, 25, and 26. Controls for pH near 5 and 6 are also included; the controls at pH 7 and 8 for acetate and EDTA were checked and shown to result in negligible reduction of the metabolism rate. At equal dilution and pH, the toxicities of the three types of leachates were greatest for acetate, followed by EDTA and then distilled water. However, if the influence of the complexing agent is accounted by substrating the relative activity of the controls then the greatest toxicity is associated with EDTA, followed by distilled water and then acetate. This is probably due to the strong complexing of the released metals by acetate.

For each leaching solution there is a trend of increasing toxicity with decreasing pH. This would be expected since metals are known to be less adsorbed at lower pH's and should then be in greater concentrations in the leachate.

At pH 6 and small dilutions, both biostimulation was observed in certain cases. The reason for this is not known although the generation of trace metal nutrients is most probable.

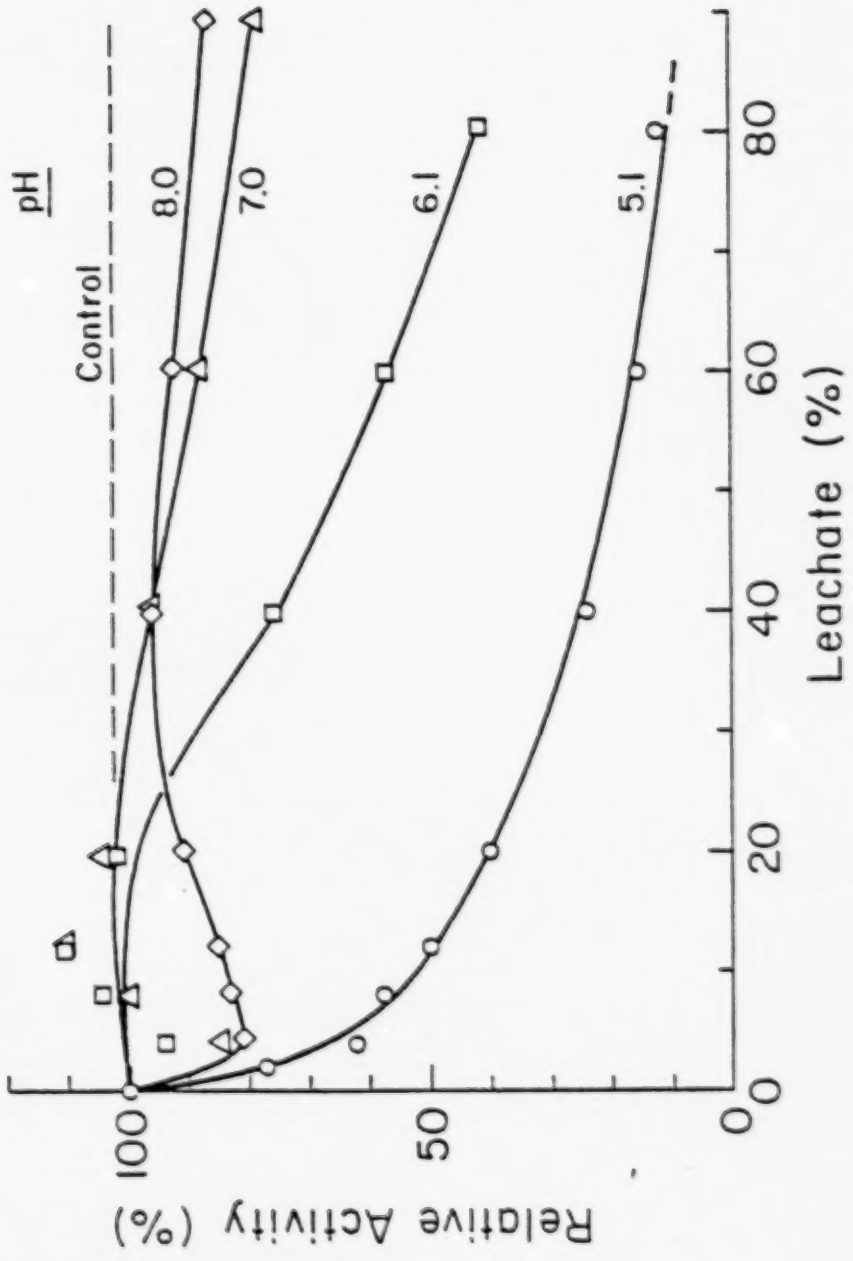

Figure 24. Aqueous Leachate Toxicity.

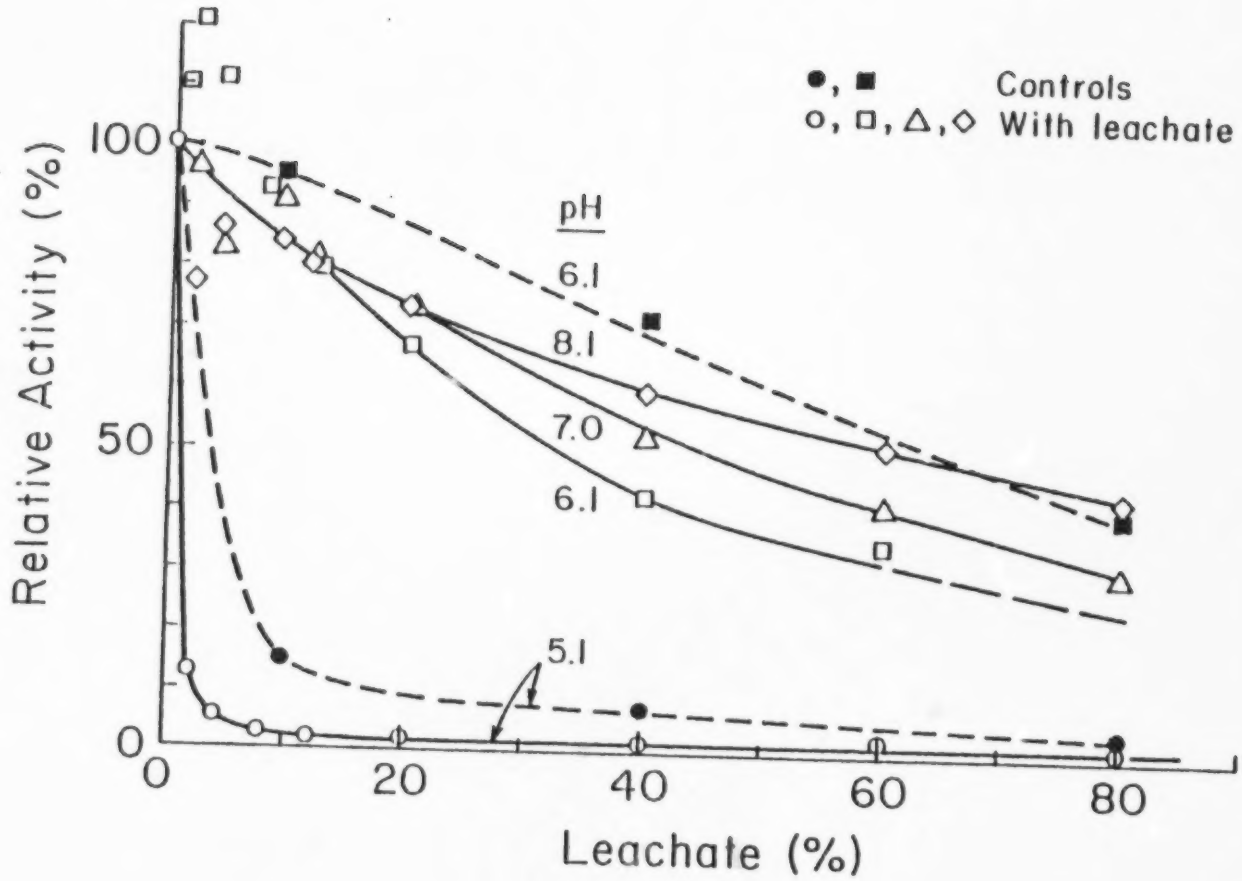

Figure 25. Acetate Leachate Toxicity, pH = 5.0.

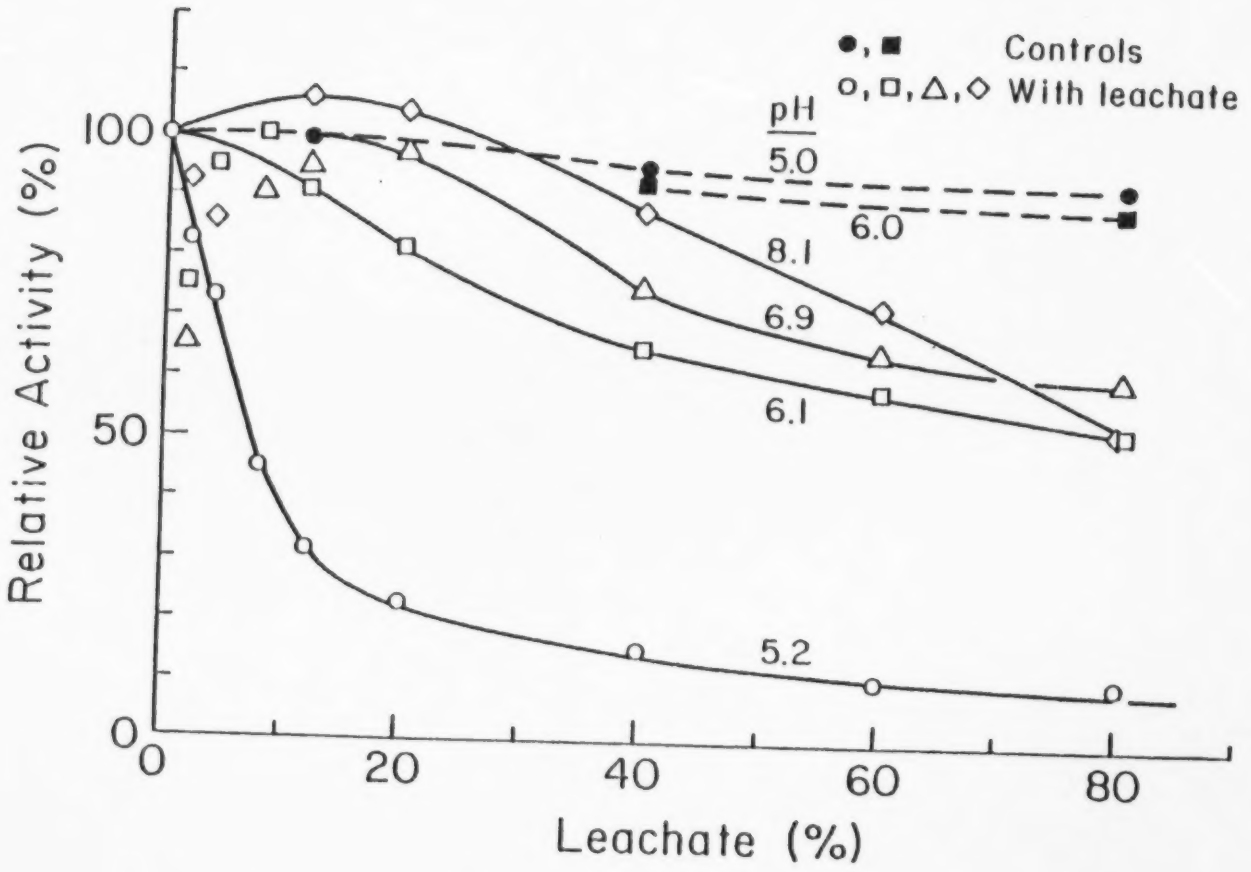

Figure 26. EDTA Leachate Toxicity, pH = 5.2.

SECTION 10

CONTINUOUS-FLOW BACTERIAL BIOASSAY

A need exists to develop a continuous bioassay to assess toxicity in wastewater effluents in streams and rivers. Several investigators (111-113) have developed such systems using fish; however, these systems are complex, expensive and offer little potential for wide-scale use.

Poels (111) lists eight criteria for a continuous monitoring system as:

1. reasonable sensitivity to a wide number of toxicants,
2. continuous and automatic measuring,
3. avoidance of false alarms,
4. stability of organisms without toxicant,
5. no effect of external changes,
6. continuous flow of water,
7. easy availability of bioassay organisms, and
8. simple monitoring.

It was judged that these criteria could be met with the Nitrobacter bioassay using a nitrate specific ion electrode to monitor metabolism rates. In this section, the development of a continuous-flow bioassay is described and the results using 2,4,5-trichlorophenol (TCP) and cadmium.

PROCEDURE

The design of the continuous bioassay system relies upon the ability of Nitrobacter to convert nitrogen in the form of nitrite (NO_2^-) to nitrate (NO_3^-); ion electrode (Orion Model 93-07) was chosen to continuously monitor the nitrate concentration. Response of the organism to toxicants was determined by monitoring the amount of conversion, which is directly related to the metabolic rate.

The continuous-flow system includes 1 liter containers for the feed and toxicant solutions, a Masterflex (No. 7014) peristaltic pump, a bacterial support column, a holder for the NO_3^- and reference electrodes, and a meter

for measuring the electrode potential in millivolts (Figure 27). A glass tube (2.0 cm by 14 cm) full of polyethylene beads was used as the bacterial column. A plug of glass wool was placed in the tube to prevent washout of beads and bacteria. Two functions were served by the beads: attachment sites for the organisms, and dispersion of the incoming flow sufficiently to approximate plug flow conditions. The probe holder was machined from a Plexiglas block; it allowed contact between the electrodes and the flowing solution. O-ring seals around the electrodes prevented leakage.

Procedures outlined in "Standard Methods" (34) (Method 419B) and the Orion Instruction Manual (114) were used to calibrate the detection system. Sodium sulfate (Na_2SO_4) was used as the ionic strength adjustor instead of the recommended ammonium sulfate because the ammonium ion inhibited the metabolism of the organisms. Calibration curves were prepared before each bioassay test.

Sodium nitrite ($NaNO_2$) at a concentration of 30 mg NO_2^--N/ℓ was used as feed. Toxicants or wastewaters were mixed with the feed solution to produce varying concentrations for testing. The 2,4,5-trichlorophenol was dissolved in a small quantity of alcohol before dilution with feed. The pH of the feed solutions was maintained between 6.5 and 7.0 with acid or base, within the optimum range for the growth of nitrifiers. Dissolved oxygen levels in the feed were maintained near saturation. The ambient temperature maintained the system temperature between 25 and 30°C.

Bacteria were dispersed throughout the beads in the column at rates slow enough to permit attachment of floc to the beads. Approximately 500 to 800 mℓ of bacterial solution were applied to the column to yield sufficient bacteria for a detectable conversion rate.

With the electrodes in-line, the bacteria were fed at a constant rate (between 2.5 and 3 mℓ per minute) with the peristaltic pump. Once the production of NO_3^- reached a steady state, the input line was switched to the bottle containing a known concentration of toxicant. The response of Nitrobacter to the toxicant was monitored in terms of nitrate production over time. If a decrease in NO_3^- resulted, a return to the regular feed solution indicated whether the toxicant was inhibitory or lethal. Results of the tests were evaluated by plotting the decrease in nitrate-nitrogen concentration versus time. Dose-response curves were developed based upon the degrees of inhibition at different toxicant concentrations.

RESULTS

Two known toxicants (2,4,5-trichlorophenol and cadmium) were used to determine the sensitivity of the continuous-flow Nitrobacter bioassay system. Since these toxicants were diluted with tap water, no interfering anions were present for the NO_3^- electrode.

Bioassay tests were completed at 2.0 and 4.0 mℓ of trichlorophenol (TCP); results of these tests are presented in Figure 28. Decreases in

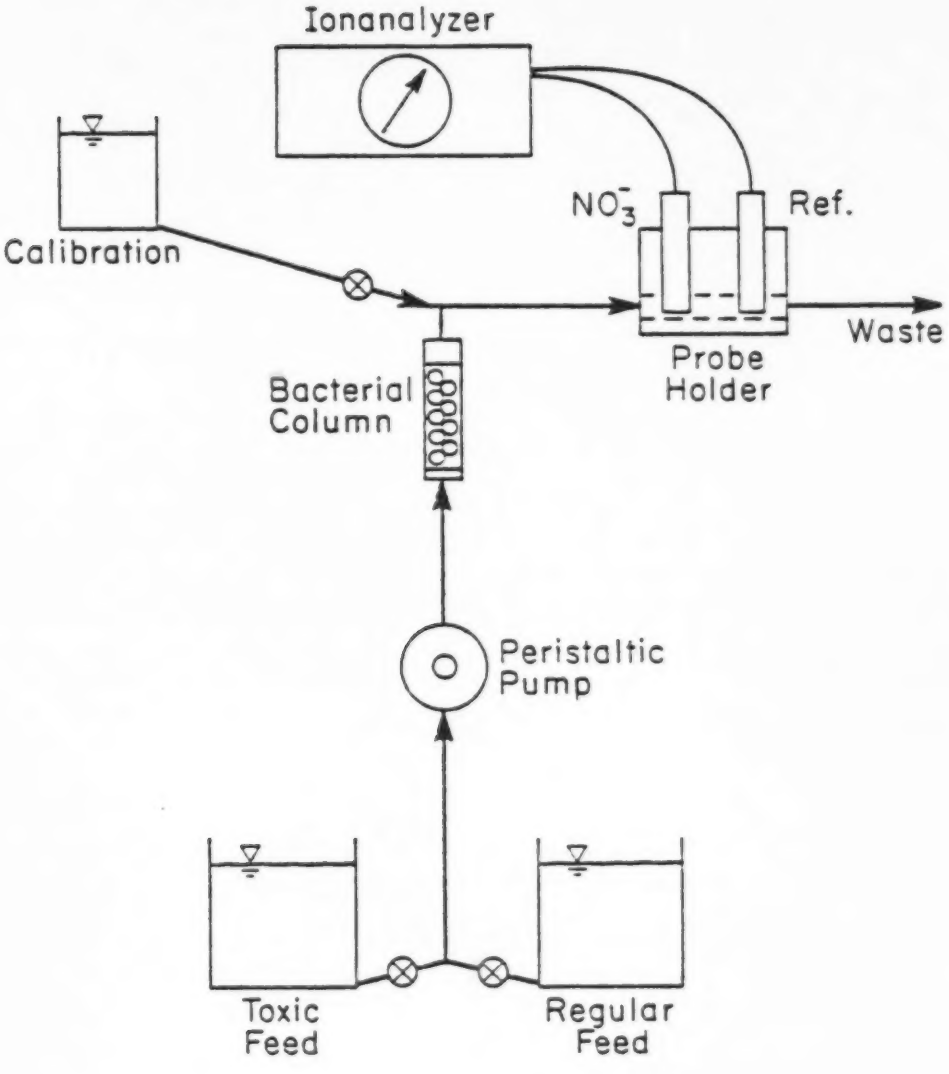

Figure 27. Continuous Flow Bioassay Apparatus.

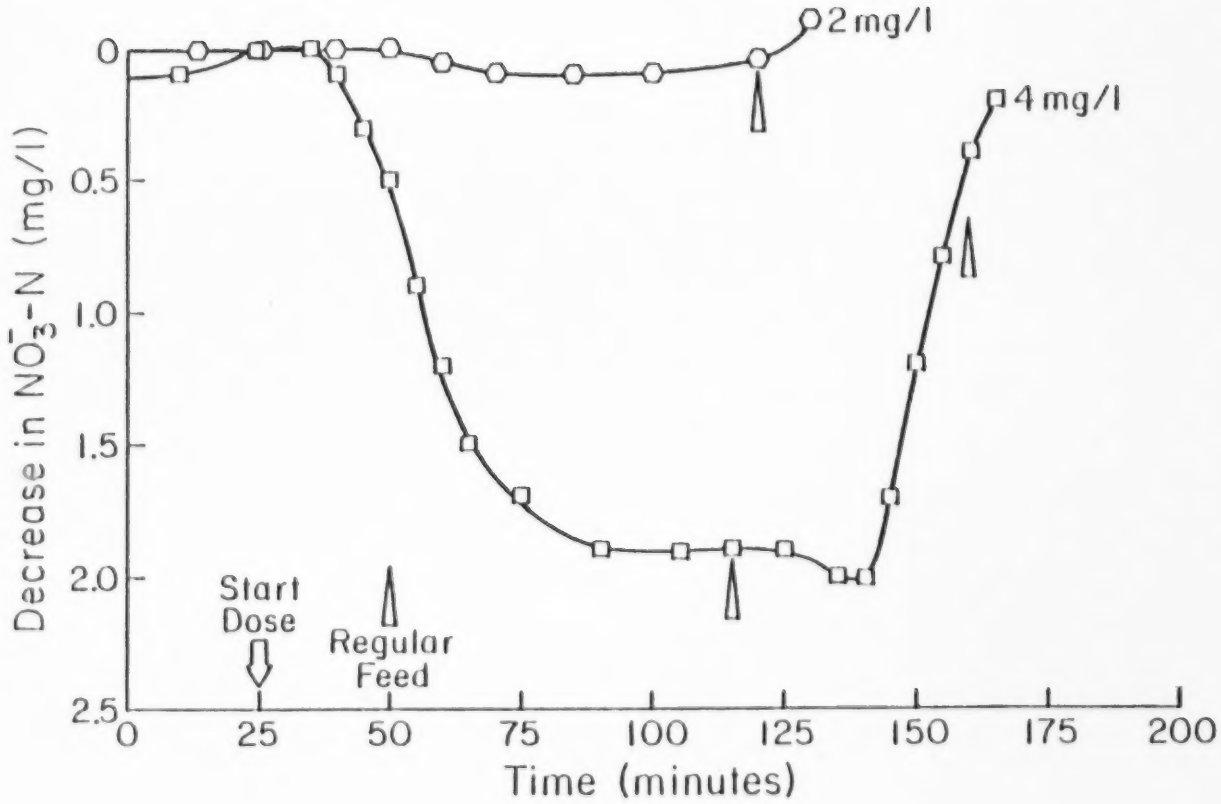

Figure 28. Effects of TCP on the Metabolism of Nitrobacter (triangles represent re-start of regular feed)

nitrate-nitrogen (NO_3^--N) produced by Nitrobacter are plotted over time to evaluate the effects of varying levels of TCP. Arrows indicate when the toxic feed was added and when the system was returned to regular feed; stable conversion rates (NO_3^- concentration) were obtained before switching feeds. Data for the plots were generated by monitoring the meter readout in millivolts at specified time intervals (approximately 5 minutes). Millivolt readings were converted to nitrate as nitrogen (mg/ℓ) with the calibration curve. These results are compared with batch test data (10) in Figure 29.

The results for cadmium (as $CdSO_4$) in the continuous-flow situation are shown in Figure 30 for 10, 50, 500, and 2000 mg/ℓ. The dose-response curve is shown in Figure 31, along with the results from batch tests. The relative metabolism rate for the continuous-flow system was determined by dividing the stable conversion rate after addition of toxicant by the rate before staring the dose. The conversion rates were corrected for the small interference (equivalent to approximately 2 mg/ℓ NO_3^--N) due to the presence of nitrite in the feed solution.

Interfering Anions Present

Often aqueous solutions will contain anions that interfere with the NO_3^- electrode; known interfering anions include ClO_4^-, I^-, NO_2^-, Br^-, and Cl^-. As a result, monitoring toxicity if these anions are present cannot be done as described for TCP and Cd^{++} above. Efforts then were focused on developing a method for assaying the toxicity of wastewaters containing interfering substances.

The alternative method for monitoring toxicity was conceived as follows. Instead of monitoring the decrease in nitrate produced by toxicant addition, the response of the system after returning to regular feed was evaluated. The response and recovery of the bioassay system when exposed to an interfering anion alone and in the presence of 500 mg/ℓ of cadmium is shown in Figure 32. With the cadmium present the NO_3^--N concentration in the effluent decreased compared to the initial steady-state concentration after the toxicant and the interfering anion are removed.

DISCUSSION

Results of the bioassay demonstrated that the continuous-flow system can detect toxicity to the Nitrobacter test culture. In addition, tests showed that the system can be applicable to monitoring wastewater toxicity in the presence of electrode interference.

Tests on TCP in the continuous-flow bioassay showed little toxicity at levels below 2 mg/ℓ, but significant toxicity was recorded at 4 mg/ℓ (Figure 28). A return to regular feed at the end of each test demonstrated the ability of the organisms to recover from a short-term exposure. These results were comparable with Nitrobacter batch bioassays on TCP (Figure 29).

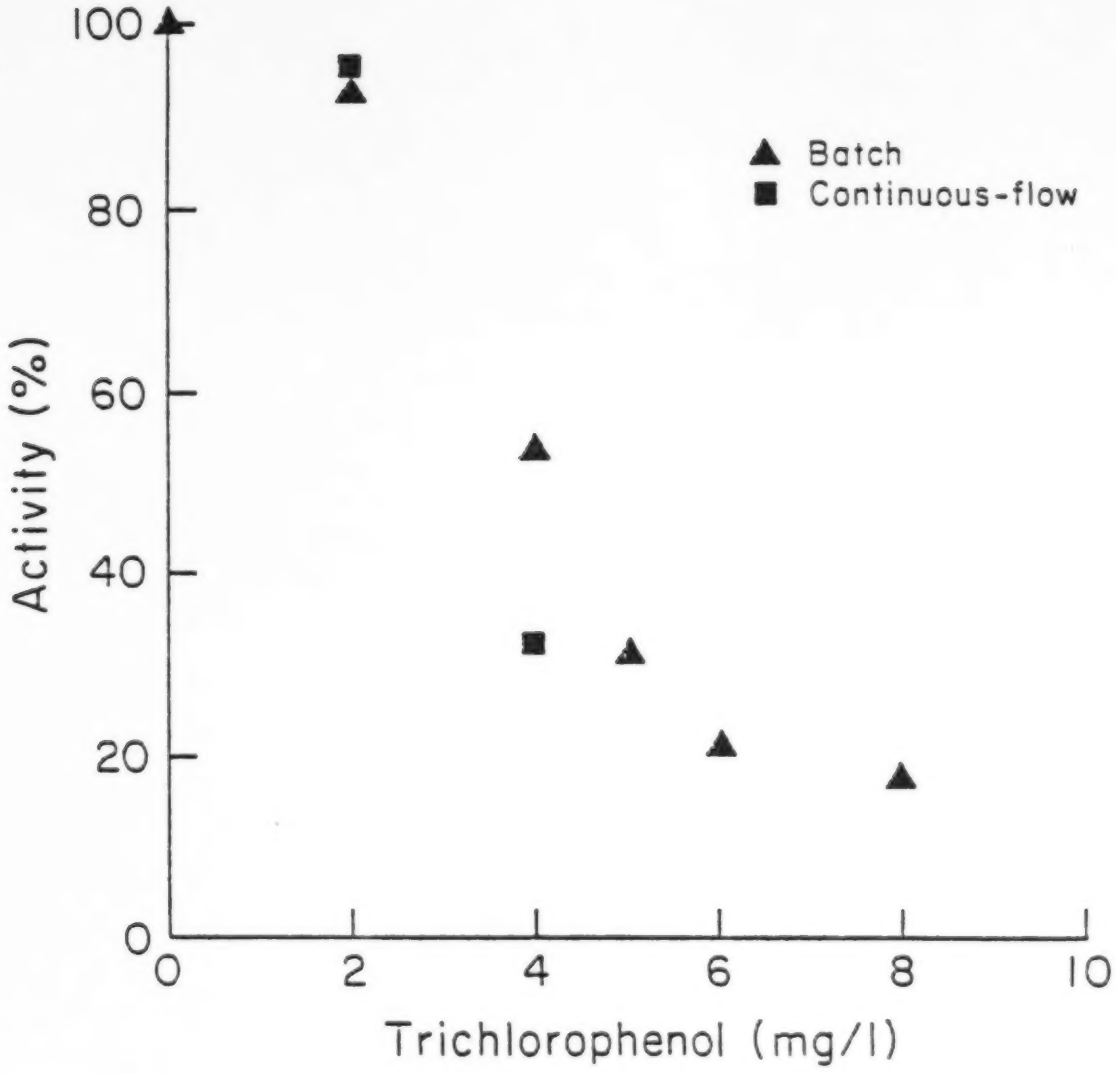

Figure 29. Comparison of Batch and Continuous Flow Toxicity of TCP.

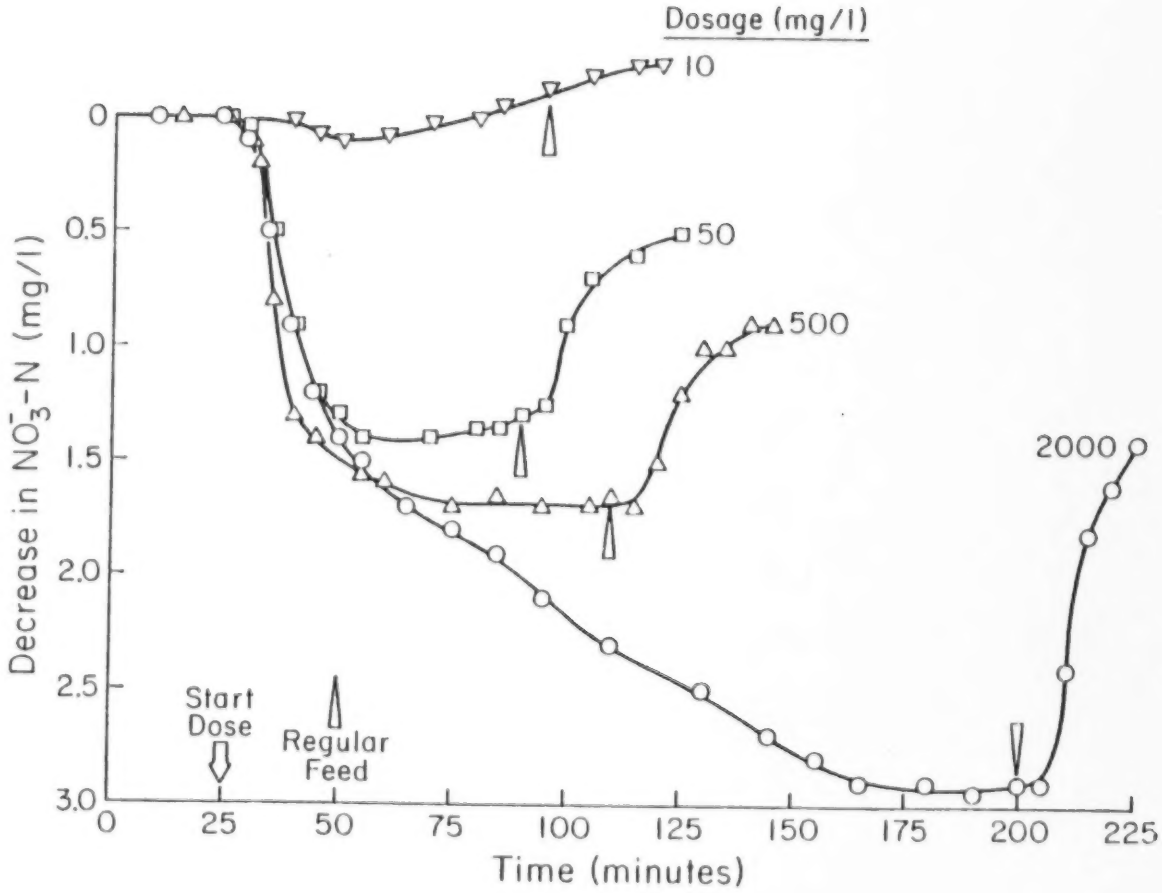

Figure 30. Effects of Cd^{2+} on the Metabolism Rate of Nitrobacter (triangles represent time at which regular feed re-started)

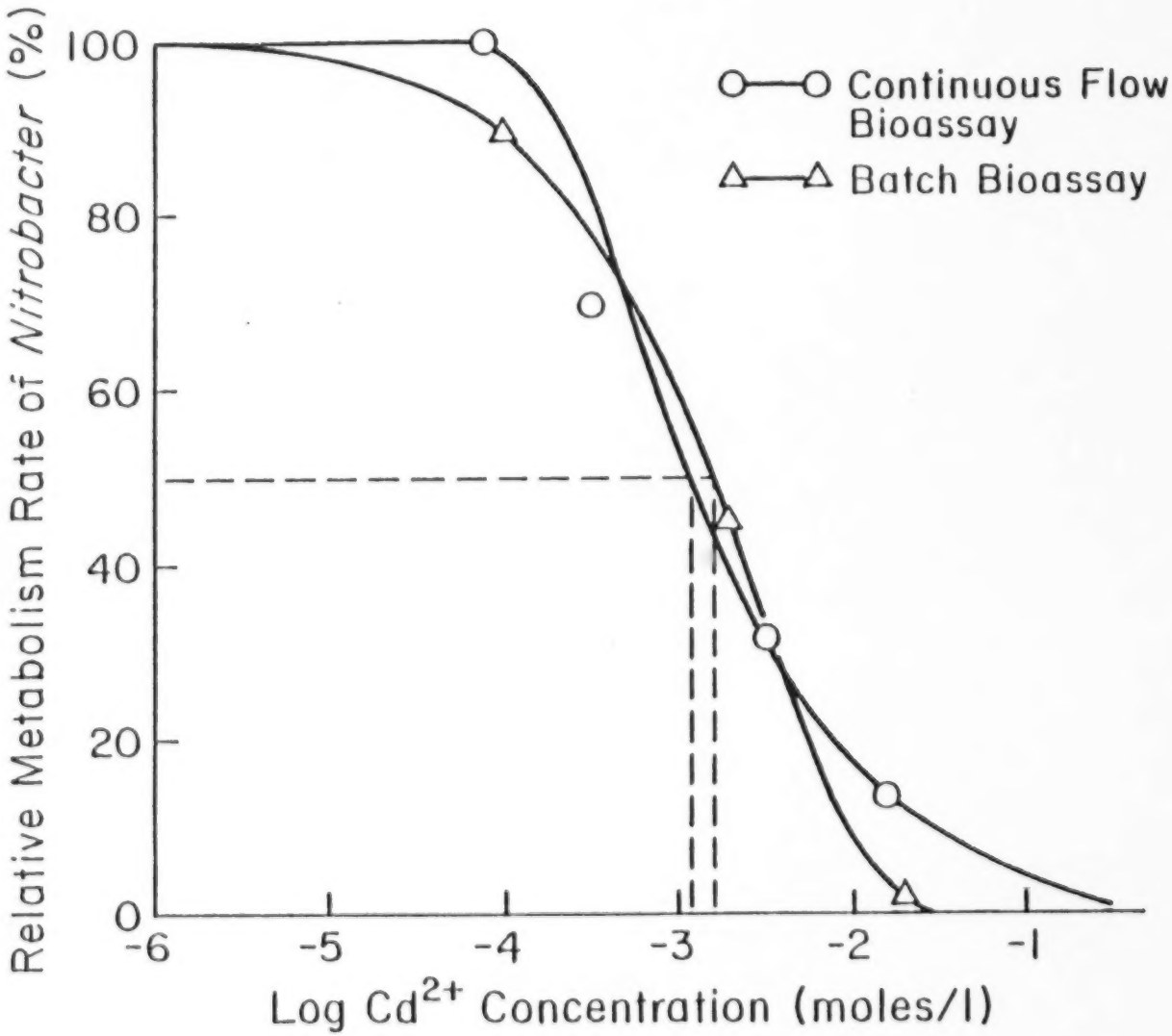

Figure . Relative Metabolism Rate of Nitrobacter versus Cd^{2+} Concentration.

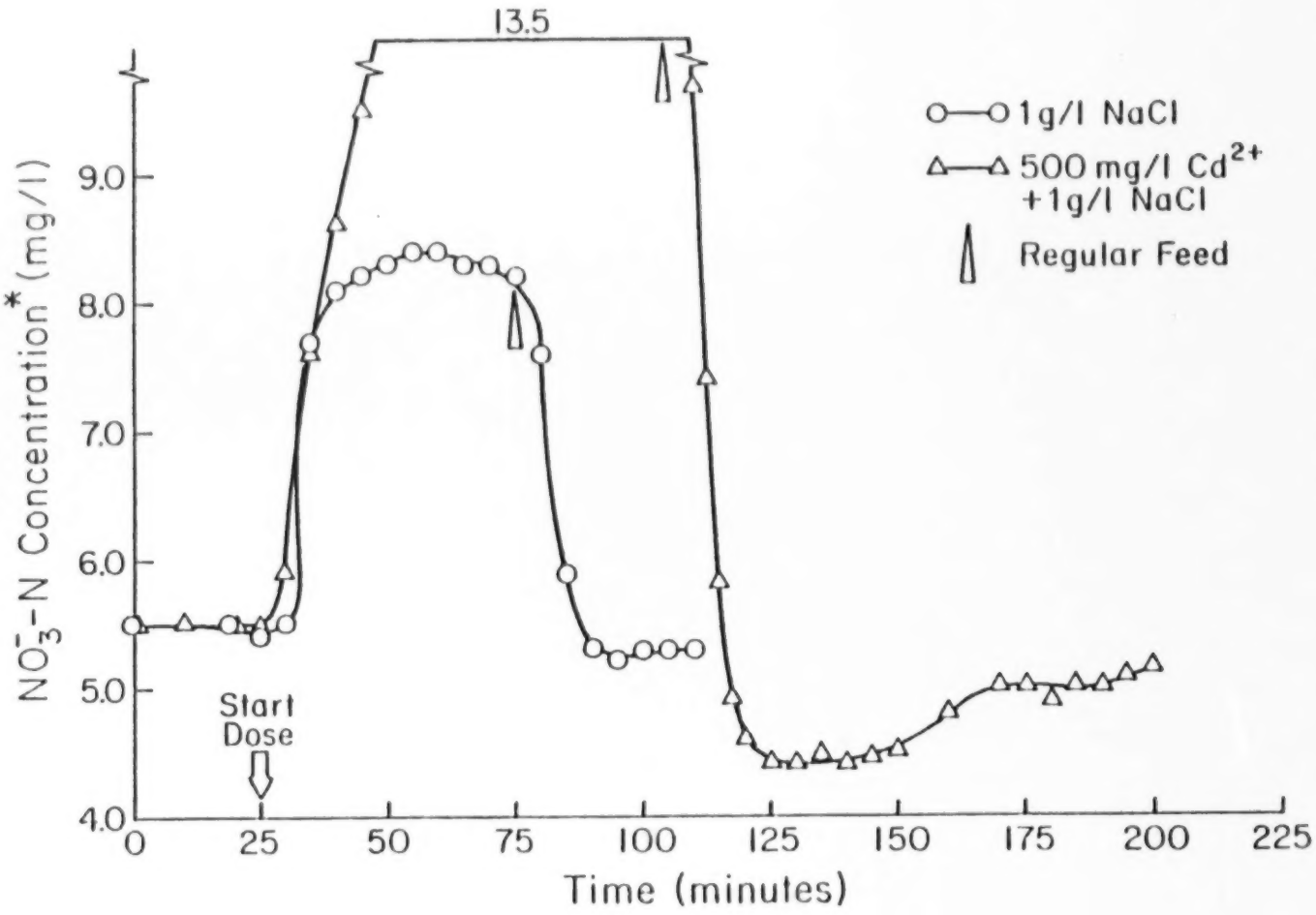

Figure 32. Monitoring Toxicity in the Presence of an Interfering Anion (*Based on Electrode Reading). (triangles represent re-start of regular feed)

The response of the Nitrobacter test culture to cadmium indicated little or no toxicity at 10 mg/ℓ, and relative increases in inhibition up to 2000 mg/ℓ which caused nearly complete inhibition. Decreases in NO_3^--N were caused by Cd^{2+} concentrations of 50 mg/ℓ and greater (Figure 30). Return to straight feed solutions showed that none of the short-term doses were lethal.

The plot of the relative metabolism rate of Nitrobacter when dosed at different cadmium concentrations (Figure 31) facilitated comparison of toxicity data for the continuous-flow bioassay with results of batch bioassays. These data indicated that the continuous-flow bioassay can detect toxicity at a sensitivity similar to that obtained with the batch bioassay method described in Section 5.

Feed solutions containing chlorides showed the effect of an interfering anion, with the resumption of stable conversion rates following removal. Similar feed solutions with both cadmium (500 mg/ℓ) and chlorides also produced interference, but return to regular feed showed a decrease in the conversion rate when compared to the initial rate of 5.5 mg/ℓ NO_3^--N (Figure 32). Data showed an increase in the production of NO_3^--N with time, which further substantiates a toxic response. These data showed that the continuous-flow system is capable of detecting toxicity in the presence of an interfering anion(s) by pulse loading the system with a noninterfering feed solution and testing for a change in the conversion rates before and after dosage of the toxicant with the interfering anion.

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